REMARKS

Claims 3, 11, 12, 13, 14, 17, 18, 19, 20, 22, 28, 29, 30, 31, 32, 33, 34, 35, 36, 44, 47, 48, and 49 have been cancelled. Claims 9 and 40-43 have been amended. New claims 50-57 have been added. Claims 1, 2, 4-10, 15-16, 21, 23-27, 37-43, and 50-57 are now pending in the application. No new matter has been added by amendment. Reexamination and reconsideration of the claims as amended are respectfully requested.

Claim Rejections – 35 USC § 112, second paragraph

7. The Examiner rejects claims 3 and 22 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the applicant regards as the invention. Claims 3 and 22 have been cancelled. The Examiner states that the dependent claims cited in this rejection fail to further limit the claims from which they depend. The Examiner suggests that the claims be placed in a product –by-process format. The Examiner also suggests that the claims should be drafted in terms of methods of making a plant by comprising transforming the exemplified plant of claim 2 or 21. New claims 54-57 reflect that suggestion.

8. The Examiner rejects claims 18-20 and 47-49 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the applicant regards as the invention. The Examiner states that the term "single gene conversion" is used contrary to its accepted meaning. Claims 18-20 and 47-49 have been cancelled and new claims 50-57 have been added. The term "single gene conversion" is no longer used in the claims. It has been replaced with the term "backcross conversion".

9. The Examiner rejects claims 3, 9-20, 22, 28-44 and 47-49 under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventor(s), at the time the application was filed, had possession of the claimed invention. The Applicant traverses the rejection. Claims 3, 11, 12, 13, 14, 17, 18, 19, 20, 22, 28, 29, 30, 31, 32, 33, 34, 35, 36, 44,

47, 48, and 49 have been cancelled in order to expedite prosecution. Claims 9, 37, 38, 39, 40, 41, 42, and 43 have been amended. New claims 50-57 have been added.

The Examiner rejects claims 9-11 which are to the F1 hybrid plant. The Applicant points out that on page 41 of the application Table 3 contains mean values of F1 plants made with PH6WG as one parent and numerous other maize inbreds as the second parent. On pages 42-46 of the application Tables 4A-4C contain mean values for traits from an F1 made with PH6WG as a parent and inbred PH581 as the second parent. The Examiner also states that the U.S. Court of Appeals Federal Circuit's decision states that "a showing of 'possession' is ancillary to the *statutory* mandate that the specification will contain a written description of the invention and that a showing of possession alone does not cure the lack of written description of the specification, as required by statute." Applicant points out that not only possession is shown via Tables 3 and 4A-4C but the requirement of written description is fulfilled by the fact that a seed deposit is made and F1 plants made with PH6WG as a parent will contain all of the homozygous alleles of PH6WG. Further, one of ordinary skill in the art would know how to cross PH6WG with another maize plant. The F1 hybrid seed and plant produced using PH6WG, regardless of the other maize plant used, is identifiable because it will have one set of alleles coming from PH6WG. One of ordinary skill in the art would be able to run a molecular profile on PH6WG and the F1 hybrid and be able to identify the F1 hybrid as being produced from PH6WG. Seed pericarp tissue, which is solely maternal in origin, can be used to discern the maternal or paternal origin of the allele sets if necessary. See page 16 of Poethig, R.S. 1982. Maize, the plant and its parts. In: W.F. Sheridan (Ed.) Maize for Biological Research, University of North Dakota Press, Grand Forks, ND. pp. 9-18, submitted as Appendix A.

The Examiner rejects claims to transformed plants of PH6WG and backcross conversion plants of PH6WG. The claims to transg nic plants and

backcross conversions are in new claims 50-57. The Examiner states that, "the claims encompass any transgenic plant using Applicant's PH6WG maize plant comprising, for example, transgenes encoding transcription factors, which are known to be capable of affecting the expressing of multiple genes and expression of multiple traits within a transformed plant." The claims include the well known methods of producing backcross and transgenic conversion plants. The product by process claims are further limited by specified conversion or transgenic traits, which include the traits of insect resistance, herbicide resistance, disease resistance, waxy starch, and male sterility. The Applicant is claiming PH6WG or a limited set of plants derived therefrom that have obtained significant genetic contribution from PH6WG.

Applicant respectfully points out that examples of transgenes, genes, and traits that can be backcrossed into the PH6WG are given in the application on page 21, lines 16-34, and also on page 23, line 20, through page 33, line 4. In order to expedite prosecution new claims 51 and 55 list the type of traits that may be conferred by backcross conversions and transgenes. Claim 51 also specifies that PH6WG is used at least twice as a recurrent parent in the development of a backcross conversion plant. Breeders, by using molecular markers, may obtain up to 98% genome identity between the backcross conversion and the recurrent parent after two backcrosses. See Marker-assisted Selection in Backcross Breeding, Openshaw, S.J. et al. Marker-assisted selection in backcross breeding. In: Proceedings Symposium of the Analysis of Molecular Data, August 1994, pp. 41-43. Crop Science Society of America, Corvallis, OR (1994) included as Appendix B. Inbred PH6WG transformed to comprise a transgene is also easily identifiable through the use of molecular markers. The transgenic version of PH6WG would have the same molecular profile as PH6WG, with the possible exception of a marker used in the profile that is located at the site of transgene insertion. However, in this case, the plethora of other identical markers would identify the line as a transgenic variant of PH6WG. This remains true regardless of the trait conferred by the transgene.

In the specification on page 4, lines 7-13, it states, "Backcrossing can be used to transfer a specific desirable trait from one inbred or source to an inbred that lacks that trait. This can be accomplished, for example, by first crossing a superior inbred (recurrent parent) to a donor inbred (non-recurrent parent), that carries the appropriate gene(s) for the trait in question. The progeny of this cross is then mated back to the superior recurrent parent followed by selection in the resultant progeny for the desired trait to be transferred from the non-recurrent parent." The method of backcrossing genes into an inbred maize plant is well known and well understood to one of ordinary skill in the art. The method has been successfully used since the 1950's (see pages 585-586 of Wych, 1988 included in the Information Disclosure Statement). In the specification, on page 21, lines 16-34, there is a description of how to backcross traits into PH6WG, which includes the claimed traits. Examples of how one of ordinary skill in the art can transfer a gene conferring a qualitative trait into a variety through backcrossing is demonstrated by the fact that the commercial market now distributes a multitude of products produced in this manner. Such conversion lines are easily developed without undue experimentation. Poehlman et al. (1995) on page 334, submitted in the information disclosure statement, states that, "A backcross-derived inbred line fits into the same hybrid combination as the recurrent parent inbred line and contributes the effect of the additional gene added through the backcross." Wych (1988) on page 585-86, also submitted in the information disclosure statement, discusses how the male sterility trait is routinely backcrossed into an inbred line and how this is used to produce a sterile/fertile blend of an F1 hybrid in order to reduce seed production costs. In fact, many commercial products are produced in this manner, and those of ordinary skill in the art consider the F1 hybrid produced with the male sterile (backcross conversion) inbred to be the same variety as the F1 hybrid produced with the non-backcross conversion inbred.

As a result of the repeated use of the recurrent parent, the backcross conversion has many genetic alleles in common with the recurrent parent. Thus, genetic analysis may be used as a means of identifying the backcross

conversion. The declaration attached as Appendix C explains how genetic analysis was used to identify backcross conversion inbreds of PH6WG. The F1 hybrid made with a transgenic version or a backcross conversion of PH6WG is also identifiable by the use of genetic markers, because the hybrid would contain one set of alleles from each parent. The Applicant further points out that in a backcross conversion both parental lines are well characterized because the deposited line is repeatedly used as a parent.

The Examiner rejects claims 37-39. Claims 37-39 are directed to growing out an F1 hybrid in which PH6WG is a parent and searching for PH6WG inbred seed. Due to the imperfect process of seed production parent seed can sometimes be contained in the hybrid seed bag. This claim covers the method of searching for inbred PH6WG seed within a bag of hybrid seed. The method is clearly described in the specification on page 5, line 21 through line 7 on page 6. One of ordinary skill in the art can practice such a method without undue experimentation. The Applicant requests that the Examiner withdraw his rejection to claims 37-39.

The Examiner rejects claims 15, 16, 40, and 42. These claims are to methods of breeding with PH6WG. Applicant points out that anyone of skill in the art would know how to utilize the well established breeding methods with PH6WG. Description of such occurs throughout the specification and descriptions can also be found in introductory plant breeding books. One of ordinary skill in the art would know how to cross PH6WG with another plant. One of ordinary skill in the art would know how to self the plant produced through the cross for successive filial generations. The Applicant requests that the Examiner specifically state the basis for the rejection to the method claims.

The Examiner rejects claims 40, 41, 42, and 43. Claims 40-43 remain pending and have been amended. Claim 40 is to the method of producing a first generation PH6WG-derived hybrid maize plant. Claim 41 is to the first generation F1 PH6WG-derived maize plant produced by the method of claim 40. The first generation F1 plant is identifiable through both breeding records and molecular marker techniques. Claim 42 is to the method of selfing the first

generation hybrid PH6WG for successive filial generations. This is a basic and well known breeding methodology, and the use of this methodology with PH6WG is described in the specification on page 21, lines 1 to 15.

Claim 43 is to plants derived from claim 42 that have at least 50% of their genetics derived from PH6WG. These claimed plants are clearly described by their method of production, which requires the use of PH6WG. Such plants must be produced through the use of PH6WG and the Examiner acknowledges that PH6WG is clearly identified. Further, Applicant has added the limitation of at least 50% inheritance from the PH6WG side of its pedigree to further emphasize the significant influence of PH6WG in the claimed product. Genetic inheritance has been accepted by both courts and governmental agencies as an accurate and reliable means of identification. In paternity cases courts routinely compel genetic testing of putative fathers to establish paternity, and federal law mandates that states have laws requiring that genetic test results be admissible in such cases without the necessity for foundation testimony or other proof. 42 U.S.C. 666(a)(5)(F)(iii)(Supp. V 1999). In such cases, a child will, on average, inherit 50% genetic contribution from each parent. Similarly, the plants produced by the method of claim 42 will also, on average, inherit 50% genetic contribution from each parent.

The Examiner states that, "the disclosure must allow one skilled in the art to visualize or recognize the identity of the subject matter purportedly described". Applicant requests that the Examiner examine the sufficiency of description of claim 43 with all of its claim limitations, including the limitation that the progeny be produced by the method of claim 42, with the use of PH6WG and retaining at least 50% genetic contribution from PH6WG. One of ordinary skill in the art would know how to cross PH6WG to develop an F1 hybrid and also how to self plants derived from the cross with PH6WG. In *Ex parte Parks*, 30 USPQ 2d 1234 (B.P.A.I. 1994), the Board of Appeals stated, "Adequate description under the first paragraph of 35 U.S.C. 112 does not require *literal* support for the claimed invention. Rather, it is sufficient if the originally-filed disclosure would have

conveyed to one having ordinary skill in the art that an appellant had possession of the concept of what is claimed."

10. The Examiner rejects claims 30-32 and 47-49 under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventor(s), at the time the application was filed, had possession of the claimed invention. Applicant traverses the rejection but has cancelled claims 30-32 and 47-49 in order to expedite prosecution.

11. The Examiner rejects claims 3, 9-20, 28-44, and 47-49 under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. Applicant traverses the rejection. Claims 3, 11-14, 17-20, 28-36, 44, and 47-49 have been cancelled in order to expedite prosecution.

The Examiner states that arguments of the Applicant have not been found persuasive "because Applicant does not teach which markers to use in the identification method that would be useful for reliably identifying a maize plant as a progeny of PH6WG, and not for instance, maize plant PHR61." The Applicant has fulfilled this written description requirement through the seed deposit of PH6WG. As described in the specification, lines 8-23 on page 16, the seed deposit allows one of ordinary skill to run a molecular profile of PH6WG. One of ordinary skill in the art would know how to obtain markers useful for such a profile. For example, the Applicant refers the Examiner to the Maize DB on the world wide web at agron.missouri.edu for an extensive listing of Markers that could be reliably used for this purpose. To expedite prosecution, Applicant submits the molecular profile of inbred line PH6WG in the declaration of Dinakar Bhattramakki attached hereto as Appendix D. Further, Applicant amends the specification to include such SSR profile. Such SSR profile is not new matter, as it is an inherent feature of inbred line PH6WG, a representative sample of which has been deposited with the ATCC. For example, see Ex parte Marsili, Rosetti, and Pasqualucci, 214 USPQ 904 (1972), in which the Patent and Trademark

Office Board of Appeals held that it was not new matter to amend the structure of a compound when a more refined analytic investigation showed a corrected formula. The Board, relying on well established cases of In re Nathan et al, 51 CCPA 1059, 328 F.2d 1005, 140 USPQ 601 (1964); In re Sulkowski, 487 F.2d 920, 180 USPQ 46 (CCPA 1973); Spero v. Ringold, 54 CCPA 1407, 377 F.2d. 652, 153 USPQ 726 (1967), and Petisi et al. v. Rennhard et al., 53 CCPA 1452, 363 F. 2d 903, 150 USPQ 669 (1966) concluded that the "products described, exemplified and claimed by Appellants inherently had and have now the structure given in the amendment in question. Consequently, the changes made in this amendment do not constitute new matter. Marsili at 906. Similarly, in the present case, inbred line PH6WG inherently had and still has the SSR marker profile being added. One of ordinary skill in the art can use molecular markers to identify PH6WG, a transgenic version of PH6WG, a backcross conversion of PH6WG and the F1 plant of the transgenic version and backcross conversion of PH6WG.

The Examiner goes on to state that "claims 9-11, 13, 14, 17-20, 28-30, 32, 33, 36-39, 41, 43, and 47-49 are rejected as not enabled for the lack of written description because Applicant has not adequately taught one of skill in the art how to make and use the claimed invention." The Examiner goes on to cite Hunsperger et al., Kraft et al. and Eshed et al. and states that they teach "it is unpredictable whether the gene or genes responsible for conferring a phenotype in one plant genotypic background may be introgressed into the genetic background of a different plant, to confer a desired phenotype in said different plant. The Examiner states that, "Hunsperger *et al* teach that the introgession of a gene in one genetic background in any plant of the same species, as performed by sexual hybridization, is unpredictable in producing a single gene conversion plant with a desired trait (see, e.g., column 3, lines 26-46)." Applicant's respectfully disagree that this is what is taught by Hunsperger et al. Hunsperger et al. teaches that a gene that results in dwarfism of a petunia plant can be incorporated into other genetic backgrounds of the petunia species (See column 2, line 67 to column 3, lines 1-4). Hunsperger et al. merely discusses the

level of the expression of that gene differed in petunia plants of different genetic backgrounds. Hunsperger et al. succeeded in incorporating the gene into petunia plants of different genetic backgrounds. Therefore, Hunsperger et al. support the fact that one can introgress a specific trait into a recurrent parent through backcross conversion. Applicant's specification provides ample disclosure of starting materials such as maize inbred PH6WG, a discussion of traditional breeding methods, and examples of transgenes and naturally occurring genes that may be used in such methods. Hallauer et al. (1988) on page 472, submitted in the information disclosure statement, state that, "For single gene traits that are relatively easy to classify, the backcross method is effective and relatively easy to manage." The teaching of Hallauer relates specifically to corn breeding and corn inbred line development.

The Examiner goes on to state that, "Kraft et al. teach that linkage disequilibrium effects and linkage drag prevent the making of plants comprising a single gene conversion, and that such effects are unpredictably genotype-specific and loci-dependent in nature (see, e.g., page 323)." Applicant disagrees that the article states such points. Kraft et al. make no mention of a plant comprising a single gene conversion or the use of backcrossing. Further, Kraft et al. relates to linkage disequilibrium and fingerprinting in sugar beet, a crop other than maize. Kraft et al. state, on p. 326, first column, "The generality of our results for other crop species needs to be investigated."

It is understood by those of skill in the art that backcross conversions are routinely produced and do not represent a substantial change to a variety. The World Seed Organization, on it's web site, writes, "The concept of an essentially derived variety was introduced into the 1991 Act of the UPOV Convention in order to avoid plagiarism through mutation, multiple back-crossing and to fill the gap between Plant Breeder's Rights and patents." As determined by the UPOV Convention, essentially derived varieties may be obtained for example by the selection of a natural or induced mutant, or of a somaclonal variant, the selection of a variant individual from plants of the initial variety, backcrossing, or transformation by genetic engineering. The commercialization of an essentially

derived variety needs the authorization of the owner on the rights vested in the initial variety." International Convention for the Protection of New Varieties of Plants, as amended on March 19, 1991, Chapter V, Article 14, Section 5(c), (emphasis added). A copy of the relevant portion of the UPOV Convention and the World Seed Organization web site is attached as Appendix E.

An example of how one of ordinary skill in the art can transfer a gene conferring a qualitative trait into a variety through backcrossing is demonstrated by the fact that the commercial market now distributes a multitude of products produced in this manner. Such conversion lines are easily developed without undue experimentation. Poehlman et al. (1995) on page 334, submitted in the information disclosure statement, states that, "A backcross-derived inbred line fits into the same hybrid combination as the recurrent parent inbred line and contributes the effect of the additional gene added through the backcross."

The Examiner goes on to state that, "Eshed *et al* teach that in plants, epistatic genetic interactions from the various genetic components comprising contributions from different genomes may effect quantitative traits in a genetically complex and less than additive fashion (see, e.g., page 1815). The Applicant would like to point out on page 1816, column 1, lines 1-5 of the Eshed et al. article it states, "Recent studies that detected epistasis of selected QTL in Drosophila (Long et al. 1995), soybean (Lark et al.1995) and maize (Doebley et al.1995; Cockerham and Zeng 1996) did not show a less-than-additive trend." Emphasis added. Applicant also adds that transferring a qualitative trait does not require undue experimentation. Please note Hallauer et al. (1988) on page 472, submitted in the information disclosure statement, which states, "For single gene traits that are relatively easy to classify, the backcross method is effective and relatively easy to manage." Claim 51 has been amended to expedite prosecution. In claim 51, the genes transferred into PH6WG are now limited to the traits of herbicide resistance, insect resistance, disease resistance, male sterility, and waxy starch.

The Examiner goes on to state that, "At claims 18-20, 37-39, and 47-49, the claimed method for producing inbred PH6WG and PH6WG maize plants or

parts thereof comprising one or more 'single gene conversion' are not enabled in view of Applicant's specification because Applicant states that neither the genotypes of the breeding cross parents nor the desired genotype to be selected is known in detail and that it is not known how the genotype would react with the environment."

Applicant respectfully points out that claims 37-39 are to a method that is referred to as "chasing selfs" in the corn research industry. Please note that claim 37 reads, "A process for producing inbred PH6WG, representative seed of which have been deposited under ATCC Accession No. PTA-4530, comprising: (a) planting a collection of seed comprising seed of a hybrid, one of whose parents is inbred PH6WG *said collection also comprising seed of said inbred* ...." Emphasis added. As stated earlier claims 37-39 are directed to growing out an F1 hybrid in which PH6WG is a parent and searching for PH6WG inbred seed that was mistakenly bagged with the hybrid seed. Due to the imperfect process of seed production parent seed can sometimes be contained in the hybrid seed bag. This claim covers the method of searching for inbred PH6WG seed within a bag of hybrid seed. The method is clearly described in the specification on page 5, line 21 through line 7 on page 6. One of ordinary skill in the art can practice such a method without undue experimentation. The Applicant requests that the Examiner withdraw his rejection to claims 37-39.

Claims 18-20 and 47-49 have been cancelled and replaced with new claims 50-57. Claims 54 and 55 are to the method of transforming PH6WG and the transformed plant with listed transgenes. These claims are similar to the claims suggested by the Examiner. New claims 50-53 are to the backcross conversions of PH6WG. The Examiner states that it would be undue trial and error experimentation by one of skill in the art to produce a "single gene conversion" or as now stated a "backcross conversion" of PH6WG. Applicant disagrees with the Examiner. As previously stated in this response, the specification, IDS references, and the seed organization of UPOV, all state that backcrossing a trait into an inbred maize plant is routinely practiced.

Claim Rejections under 35 U.S.C. § 102 and 103

The Examiner states that, "Claims 14, 41, and 43 remain rejected under 35 U.S.C. 102(b) as anticipated by or in the alternative, under 35 U.S.C. 103(a) as obvious over Hoffbeck (U.S. Patent No. 5,463,173)." Applicant traverses the rejection.

Applicant has cancelled claim 14. Applicant has amended claim 41 and claim 43. Claim 41 is limited to the F1 hybrid produced from one cross with PH6WG. Claim 43 is limited to progeny produced by the method of claim 42, which requires the use of PH6WG, and is further limited to progeny deriving at least 50% genetic contribution from PH6WG.

As evidenced by the declaration of Stephen Smith submitted as Appendix F, both PH6WG and its progeny within the scope of claim 43 are distinct from PHR61 taught in U.S. Patent No. 5,463,173.

In light of the above, Applicant respectfully requests that the Examiner reconsider and withdraw the rejection to claims 41 and 43 under 35 U.S.C. 102 (b) and 103(a).

Claims 1, 2, 4-10, 15-16, 21, 23-27, 37-43, and 50-57 are now pending in the application. The amendments made herein do not in any way change the claim scope which the Applicant believes is allowable but is meant to hasten the issuance of the patent.

CONCLUSION

Applicant submits that in light of the foregoing amendments and the remarks, the claims 1, 2, 4-10, 15-16, 21, 23-27, 37-43, and 50-57 are in condition for allowance. Reconsideration and early notice of allowability is respectfully requested. If it is felt that it would aid in prosecution, the Examiner is invited to contact the undersigned at the number indicated to discuss any outstanding issues.

Respectfully submitted,
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2 MAIZE - THE PLANT AND ITS PARTS

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One of the greatest deterrents to an appreciation of plant morphology is the terminology used to describe various plant parts. This problem is compounded in the case of maize because of its relatively unusual structure. We all learn that plants have a vegetative body composed of stems, leaves and roots, and that flowers contain sepals, petals, pistils and stamens. Maize, however, has at least three kinds of leaves, two kinds of stems, two kinds of roots, and two kinds of flowers in which glumes, lemmas and paleas take the place of sepals and petals. Fortunately, these parts are arranged in a relatively simple fashion, so the task of mastering maize morphology is not as difficult as it might seem. In this article we will identify some of the most important parts of the maize plant and describe their organization. More detailed descriptions of the developmental morphology of maize have been provided by a number of investigators. Kiesselbach (1949, reprinted 1980) gives a good general picture of maize structure and development. The external morphology and the histology of the vegetative and reproductive shoots have been studied by Bonnett (1948, 1953), Sharman (1942) and Abbe and co-workers (Abbe and Phinney, 1951; Abbe et al., 1951), while the most comprehensive descriptions of the embryogeny are those of Randolph (1936) and Abbe and Stein (1954). A summary of the histology of the corn plant, written by Sass in 1955, has been reprinted in the recent edition of Corn and Corn Improvement (1976).

The organization of the plant body: Maize is a member of the grass family, the Gramineae, and as in all grasses, most of the plant body is leaf tissue (Fig. 1a). To appreciate the general organization of the maize plant it is helpful, therefore, to see it in a leaf-less state (Fig. 1b). Stripped naked, the maize plant is not very impressive. Its main stem, or culm, is a slender, segmented shaft similar to a stalk of bamboo or sugarcane. The enlarged joints along the stem, the nodes, mark the points of leaf attachment; the stem segment between nodes is called the internode. Each node bears a single leaf in a position opposite that of the neighboring leaf, giving the plant two vertical rows of leaves in a single plane (Fig. 1a; 2). This so-called distichous phyllotaxy is typical of all leaf-like appendages, wherever they occur on the plant.

Maize has unisexual, rather than bisexual flowers. Male (staminate) flowers are located at the apical tip of the main stem in the tassel, a branched inflorescence. Female (pistillate) flowers are found in one to several compact ears, located on the ends of short branches near the middle of the stem (Fig. 1b; 2).

This partitioning of male and female flowers in separate structures distinguishes maize from other cereals and is one of the principal reasons that its genetics has been so conveniently explored. Making controlled pollinations in maize requires little more effort than that involved in placing a bag over the tassel and ear shoot. To perform a controlled pollination in rice, wheat, barley and other cereals, it is necessary to emasculate each

Appendix A

flower used as a female parent, an especially tedious job when each flower yields only one seed.

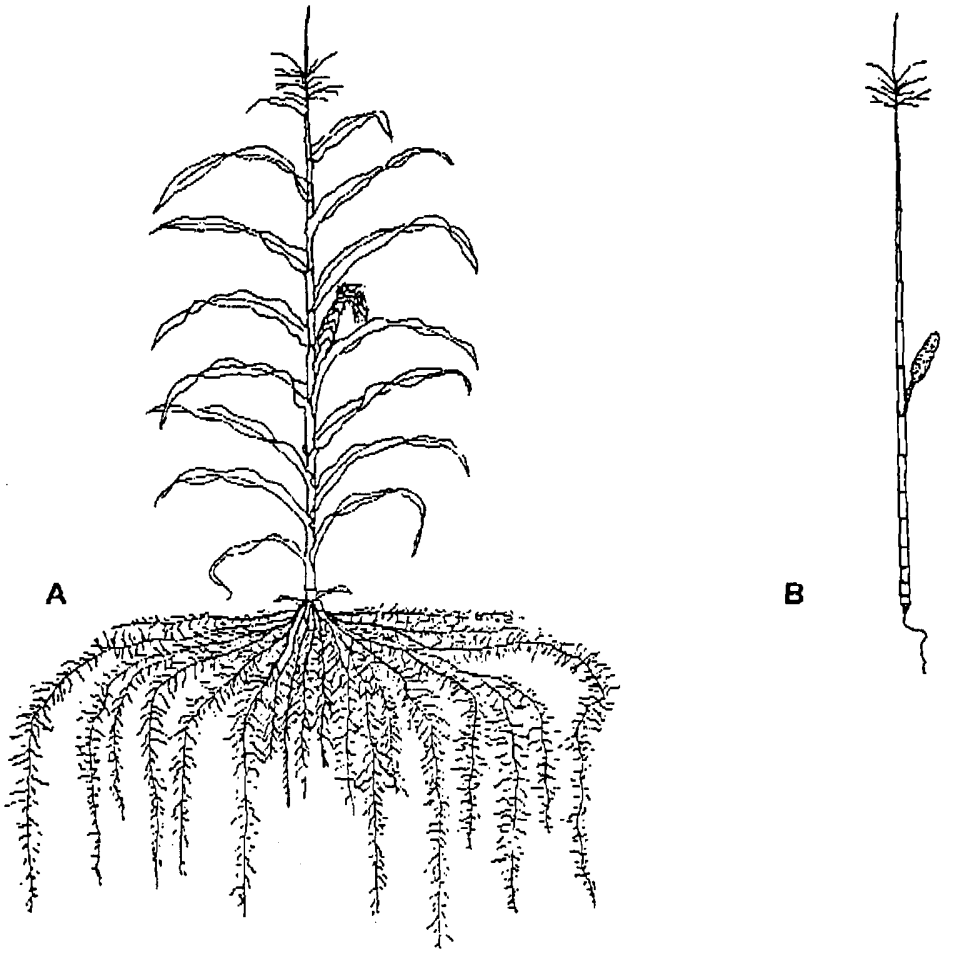

Figure 1. a) Mature maize plant (after Kiesselbach, 1949). b) Mature maize plant drawn without leaves and adventitious roots. The apical end of the main stem (culm) terminates in the tassel, while the basal end terminates in the primary root (radicle). The ear shoot arises from an internode near the center of the culm.

Maize also differs from closely related species in that it has relatively few branches. Only the lower 10 to 12 internodes of the stem produce branch primordia, and most of these remain suppressed. Above-ground primordia develop into ear shoots, while those located at subterranean internodes develop into tillers--branches identical in structure to the main stem. Commercial hybrids (except sweet corns) generally tiller very little, and typically produce a single viable ear shoot. In contrast, some "varieties" may have several large tillers and may produce 2 ears on the main stem and some ears on tillers.

The stem: During the first four weeks after germination, the growing point of the stem lays down all the nodes and internodes of the plant and then differentiates into a tassel. At the time of tassel formation the stem is not more than 3-4 inches tall, even though the plant may be 3-4 feet in

height (Fig. 3). Subsequently, the stem begins to elongate rapidly, with most of the growth occurring at the base of the internodes. The lowermost 6-8 internodes do not participate in this growth, however, and remain below ground where they produce the root system and tillers. These subterranean internodes taper sharply towards the base of the stem, forming a distinctive region, the crown (Fig. 1b). The stem is thickest a few inches above ground, and tapers gradually towards the tassel. All the internodes from the top ear downward have a distinct groove associated with the axillary bud at the base of the internodes; internodes above the ear lack axillary buds and are smoothly cylindrical.

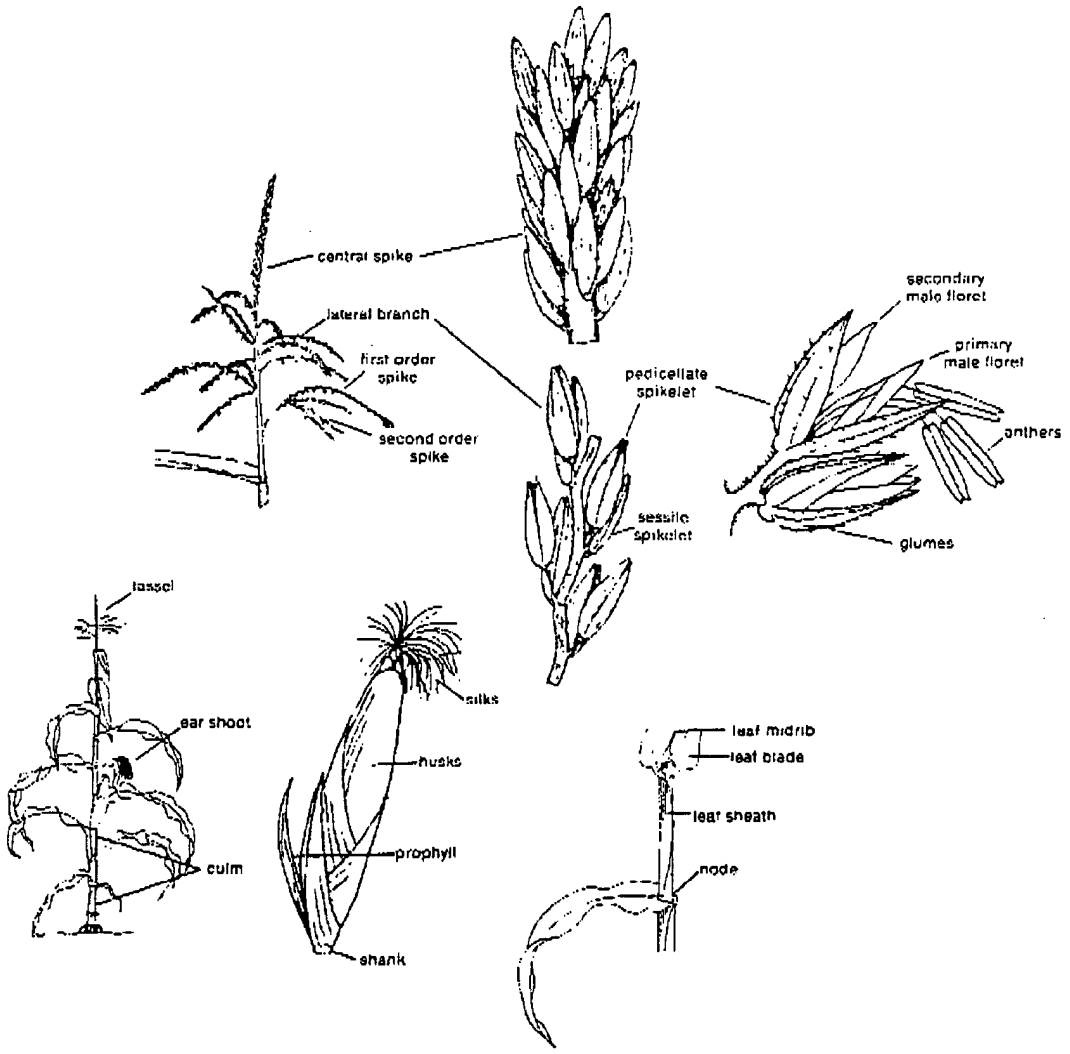

Figure 2. The major parts of the maize plant. Drawings in part from P. Weatherwax in Corn and Corn Improvement, 1955, and E. D. Styles et al. in Can. J. Genet. Cytol. 15:59, 1973; figure assembled by M. M. Johri and E. H. Coe.

The stem of an ear shoot, called the shank (Fig. 2), differs from the main stem in being relatively short in most strains. In addition, the internodes of the shank are variable in number, irregular in shape and size, and tend to have a crinkled rather than smooth surface. Secondary ear shoots commonly occur on the shank of several types of maize, but are rare in most commercial strains unless fertilization of the apical ear is prevented.

Figure 3. A four week old plant (approximately 3 feet tall) in which the stem apex has differentiated into a tassel. As shown on the right, the stem is still relatively short at this stage.

The tassel: The tassel, located at the top of the culm, consists of a series of large branches (spikes) covered with numerous, small flower-bearing branches (spikelets: Fig. 2) Each branch point on a spike bears two spikelets, one on a long stem (pedicellate), the other on a short stem (sessile) (Fig. 4a). Each of these spikelets, in turn, produces two functional florets. Although tassel florets contain both stamens and a pistil, the pistil normally degenerates soon after it is initiated, making the floret functionally male. However, pistils will develop at the base of the tassel under some environmental and physiological conditions, and are quite common on tillers.

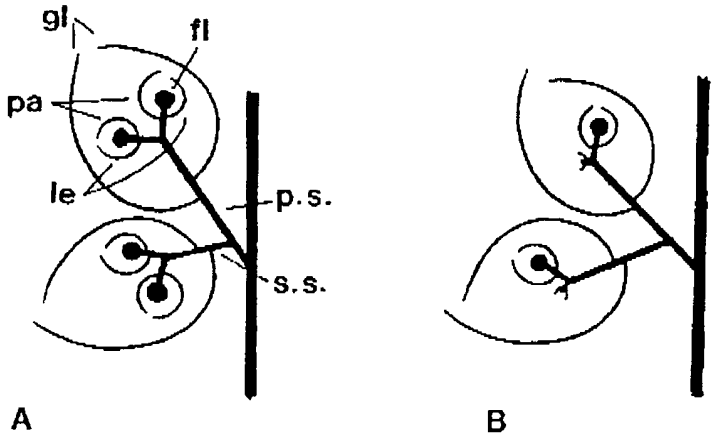

Figure 4. Schematic drawing of a pair of tassel spikelets (A) and a pair of ear spikelets (B). Note that the lower floret in the ear spikelet aborts early in development. p.s. - pedicellate spikelet; s.s. - sessile spikelet; gl - glumes; le - lemma; pa - palea; fl - floret.

Surrounding both florets on a spikelet are 2 leaf-like scales called glumes (Fig. 2; 4a). Within the glumes, each floret is individually enclosed in another pair of scales, one located adjacent to the glume (the lemma), the other located between the two florets (the palea) (Fig. 4a). At anthesis, these scales are forced apart by the swelling of conical structures (lodicules) at the base of the 3 stamens, and the filamentous base of the stamens elongates, forcing the anthers out of the flower (Fig. 2). As they dangle downwards, the anthers shed pollen from openings at their tip.

Pollen grains are the multicellular products of the haploid microspores that result from the meiosis of a microspore mother cell (microsporocyte). Meiosis takes place in the anther before the tassel emerges from the leaf sheaths. After meiosis, the 4 resulting haploid microspores separate from each other, and each forms a thick wall. Shortly before shedding, each microspore undergoes two mitotic divisions. The first division is asymmetric, and produces a relatively large vegetative cell and a smaller generative cell. In the second division, the generative cell divides to form two sperm cells.

The ear: The ear is morphologically similar to the tassel, although this resemblance is obscured by differences in the relative size of their parts. The crucial difference between them is, of course, that the tassel contains male flowers, and the ear bears female ones. This difference is due simply to the fact that during the formation of an ear floret, stamen primordia are arrested at an early stage in their development, while the pistil develops fully. Each functional ear floret has a single ovary, which terminates in an elongated style, or silk (Fig. 5). Within the ovary is a single embryo sac. The embryo sac is the product of one of the four haploid cells resulting from the meiosis of the megaspore mother cell. While its three sister cells degenerate, the nucleus of this cell divides three times to produce 8 haploid nuclei within a common cytoplasm (the embryo sac). Two of these nuclei (polar nuclei) migrate to the center of the embryo sac where they become closely associated. The three nuclei remaining at the base of the embryo sac

subsequently undergo cellularization to form the egg cell and two synergids, while the 3 nuclei at the tip of the embryo sac proliferate to form 24-48 antipodal cells.

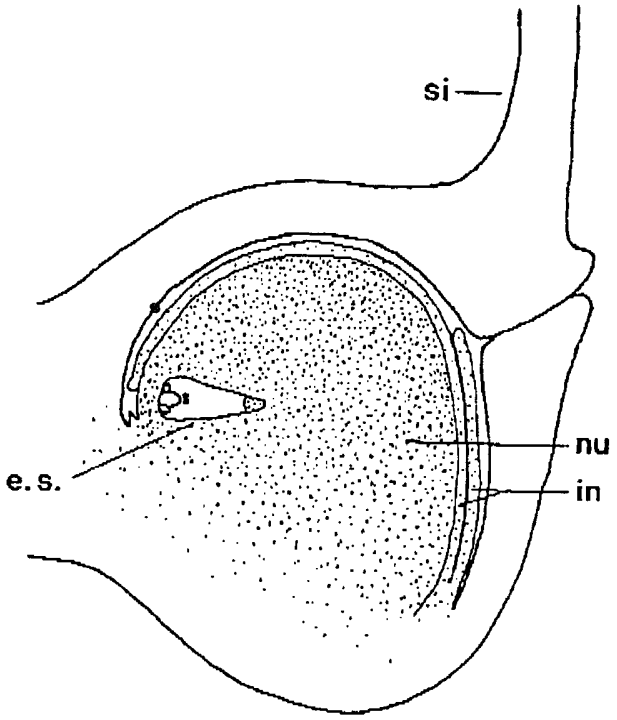

Figure 5. Radial longitudinal section of an ovary with an unfertilized embryo sac (after Randolph, 1936). Upon fertilization, the nucellus is digested by the expanding embryo sac and the tissue surrounding the nucellus is transformed into the pericarp. si - silk; e.s. - embryo sac; nu - nucellus; in - integuments.

The ear also differs from the tassel in that it has no major lateral branches. Its thick, lignified axis, the cob, is homologous to the central spike of the tassel. As in the tassel, ear spikelets come in pairs, but in the ear they are equal in size and only one of the florets in each spikelet is functional (Fig. 4b). An ear therefore has an even number of parallel rows of equally sized kernels equal to the number of spikelets on the cob. The number of rows (or ranks) of kernels ranges from 4 to 30.

The glumes, lemmas and paleas of the ear spikelets are readily visible in an unfertilized ear, but are soon obscured by the enlargement of the ovary after fertilization. In a mature ear these structures are represented by the chaff that adheres to the cob and the base of the kernel after it is shelled.

The leaf: Maize produces three kinds of vegetative leaves: foliar leaves, husk leaves and prophylls. A foliar leaf is located at each of the nodes on the main stem, husk leaves are located on the shank of the ear shoot, and prophylls are found at the base of the shank between the ear shoot and the stem (Fig. 2).

The foliar leaf has two distinct parts--the blade, a flat portion extending away from the stem, and the sheath, a basal part that wraps tightly around the stem (Fig. 2). Internally, the blade consists of a spongy network of cells traversed by a series of parallel, longitudinal veins. This flexible lamina is supported by the midrib, a thickened, translucent structure located in the center of the leaf. The sheath is thicker and more rigid than the blade, possesses fewer longitudinal veins, and lacks a prominent midrib. The sheath completely encircles the internode above the node to which it is attached and may extend the entire length of that internode. During the early development of the plant, the leaf sheaths provide most of the mechanical support necessary to keep the stem upright. At the boundary between the blade and the sheath there is a distinct hinge of translucent tissue. In this region, the leaf blade and leaf sheath narrow sharply, forming an indentation in the leaf margin. The wedge of translucent tissue adjacent to this indentation is known as the auricle. The ligule is the thin collar of filmy tissue located on the inside of the hinge.

The husk leaves surrounding the ear are usually considered modified leaf sheaths, with vestiges of the blade portions occasionally present. In some strains husk leaves develop a prominent ligule and leaf blade. In contrast to the leaf sheath, husk leaves are relatively thin and flat. Each husk leaf is attached to a unique node on the shank, and all but a few upper ones are arranged distichously.

Located between an ear shoot and the stem, the prophyll looks superficially like a husk leaf, but is distinguished by having two keels (midribs) and a split apex. These features suggest that the prophyll arose evolutionarily from the fusion of two foliar leaves. The homology of the prophyll is still controversial, however. Galinat (1959), for example, considers the prophyll one of the basic units of maize morphology, the others being the internode, leaf and axillary bud.

The root: More is known about the growth, cell biology, physiology and anatomy of the primary maize root, or radicle, than perhaps any other organ of the plant. Its histological structure, described by Sass (1976) and Kiesselbach (1949), is typical of roots in general. The apex of the root is sheathed in a loose network of root cap cells. Immediately behind the apex is a zone of cell division and elongation, beyond which root hairs are initiated. Larger lateral roots arise at varying points behind the zone of root hair formation. Cell division is restricted to the apical 3 mm of the root, and occurs at a maximal rate 1.25 mm behind the apex. The zone of elongation extends 8 mm behind the apex, the rate of elongation being maximal 4 mm from the tip (Erickson and Sax, 1956). Those interested in using the root for physiological or cell cycle studies should consult Silk and Erickson (1979; 1980) and Green (1976) for an analysis of the growth parameters that must be taken into consideration in such studies.

The primary root represents the basal end of the plant axis, which in maize and other grasses contributes relatively little to the ultimate root system (compare Fig. 1a and b). Most of the root system consists of adventitious roots produced by the basal-most internodes of the stem. The primordia of a few adventitious roots are normally present in the embryo, and these emerge soon after germination. New root primordia are subsequently initiated at the base of all subterranean internodes, and also appear

at 2 or 3 above-ground internodes after the stem has elongated. Subterranean adventitious roots are sometimes called crown roots, while those initiated above ground are known as brace roots.

Adventitious roots grow horizontally for several feet before turning downwards. As a result, the root system of a single plant often covers a region 6-8 feet in diameter, while the depth of the root system may be as much as 6 feet. As it grows, the root branches profusely in the region behind the apex, forming both secondary roots and unicellular root hairs. The total length of root system of a mature plant has been estimated to be 6 miles.

The kernel: The events surrounding the process of fertilization have been described by Miller (1919), Kiesselbach (1949) and Pfahler (1975); unfortunately, ultrastructural information about this phenomenon is still unavailable.

The silk is receptive to pollen along its entire length. Within 5 minutes after a pollen grain lands on a silk it sends out a tube which penetrates the silk and grows downward towards the ovary. During this process the vegetative nucleus and the two sperm cells migrate to the tip of the pollen tube where they remain throughout its growth. Upon reaching the embryo sac, 12 to 24 hours after germination, the end of the pollen tube bursts, releasing the two sperm. One sperm nucleus fuses with the two polar nuclei in the center of the embryo sac to form a triploid cell that gives rise to the endosperm. The other sperm nucleus fuses with the egg nucleus to form the zygote. As often as 2% of the time the polar nuclei and the egg nucleus are fertilized by sperm from different pollen grains, with the extra sperm nuclei being somehow lost (Sarkar and Coe, 1971). This phenomenon, called heterofertilization, can lead to a non-correspondence between the genotype of the endosperm and embryo when the male parent is heterozygous.

The development of the kernel following fertilization has been described in detail by Randolph (1936). We will only note here that this process takes 40-50 days and is accompanied by a 1400-fold increase in the volume of the embryo sac. The growth of the embryo and the accumulation of food reserves in the endosperm is completed by about day 40, and the remaining 10-20 days is spent maturing and drying.

A mature kernel has three major parts: the pericarp, endosperm and embryo (Fig. 6). The pericarp, the tough transparent outer layer of the kernel, is derived from the ovary wall and is therefore genetically identical to the maternal parent. The endosperm and embryo represent the next generation.

The endosperm makes up about 85% of the weight of the kernel and is the food source for the embryo for several days after it germinates. This food takes the form of intracellular starch grains and protein bodies, and is concentrated to varying degrees in different parts of the endosperm (Duvick, 1961). In flint-type kernels the concentration of starch and protein bodies is higher around the periphery of the endosperm than in the center, giving the endosperm a hard, corneous external layer, and a soft, granular center. In dent kernels, the granular tissue extends to the crown of the endosperm so that it collapses upon drying and produces a distinct indentation. These two traits are polygenic in their inheritance and are

characteristic of specific races of maize. Other common endosperm traits, such as sugary, floury or shrunken, are single gene mutations and can exist in either a flint or dent background.

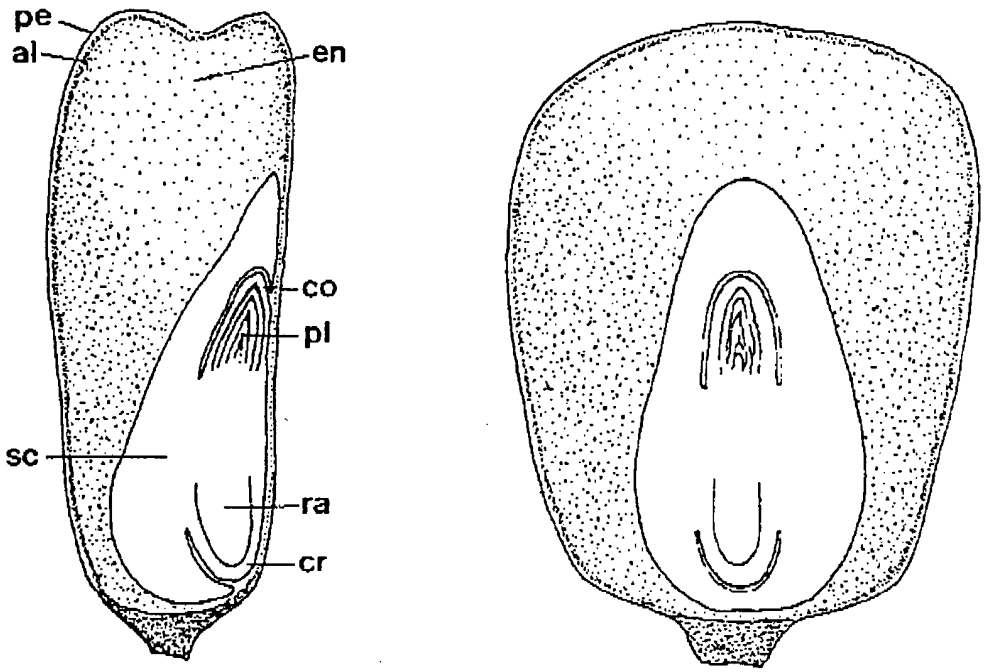

Figure 6. Longitudinal sectors of a mature dent kernel, taken perpendicular (left) and parallel (right) to the upper face of the kernel (after Kiesselbach, 1949). pe - pericarp; en - endosperm; al - aleurone; sc - scutellum; co - coleoptile; pl - plumule; ra - radicle; cr - coleorhiza.

Much of our understanding of gene action in maize is based on the analysis of genes affecting the pigmentation of the external layer of the endosperm, the aleurone. This specialized single cell layer is the only part of the endosperm capable of becoming intensely pigmented. Internal endosperm cells may be either yellow or white.

The embryo is located on the broad side of the kernel facing the upper end of the ear, beneath a thin layer of endosperm cells. Most of the tissue in the embryo is part of the scutellum, a spade-like structure concerned with digesting and transmitting to the germinating seedling the nutrients stored in the endosperm. The shoot and root axis are recessed in the outer face of the scutellum. In a mature kernel, the shoot (plumule) has 5 to 6 leaf primordia that are arrested at successive stages of development (Abbe and Stein, 1954). Surrounding the shoot is a cylindrical structure called the coleoptile. Upon germination, the coleoptile elongates until it is above ground and is then ruptured by the more rapid expansion of the rolled leaves within it. The root is enclosed in a sheath of tissue called the coleorhiza. Unlike the coleoptile, the coleorhiza does not elongate very much, and gives way to the radicle as soon as it emerges from the seed.

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Marker-assisted Selection in Backcross Breeding

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Abstract. **The backcross breeding procedure has been used widely to transfer simply inherited traits into elite genotypes. Genetic markers can increase the effectiveness of backcrossing by 1) increasing the probability of obtaining a suitable conversion, and 2) decreasing the time required to achieve an acceptable recovery. Simulation and field results indicated that, for a genome consisting of ten 200-cM chromosomes, basing selection on 40 or 80 markers in 50 BC individuals that carry the allele being transferred can reduce the number of backcross generations needed from about seven to three.**

The backcross breeding procedure has been used widely to transfer simply inherited traits into elite genotypes. Usually, the trait being transferred is controlled by a single gene, but highly heritable traits that are more complexly inherited have also been transferred successfully by backcrossing; for example, maturity in maize (Rinke and Sentz, 1961; Shaver, 1976). Today, backcrossing is being used to transfer genes introduced by such techniques as transformation or mutation into appropriate germplasm.

Several plant breeding textbooks give good descriptions of the backcross procedure (Allard, 1960; Fehr, 1987). A donor parent (DP) carrying a trait of interest is crossed to the recurrent parent (RP), an elite line that is lacking the trait. The F_1 is crossed back to the RP to produce the BC_1 generation. In the BC_1 and subsequent backcross generations, selected individuals carrying the gene being transferred are backcrossed to the RP. The expected proportion of DP genome is reduced by half with each generation of backcrossing. Ignoring effects of linkage to the selected DP allele being transferred, the percentage recurrent parent (%RP) genome expected in each backcross generation is calculated as:

$$\%RP = 100\,[1 - (0.5)^{n+1}]$$

where n is the number of backcrosses.

Backcrossing of selected plants to the RP can be repeated each cycle until a line is obtained that is essentially a version of the RP that includes the introgressed allele. After six backcrosses, the expected recovery is >99% (Table 1).

Until recently, discussions of the recovery of the RP genome during backcrossing have emphasized the expected values for %RP shown in Table 1, and have largely ignored the genetic variation for %RP that exists around the expected mean. With the development of genetic markers capable of providing good genome coverage, there has been interest in taking advantage of that variation to increase the efficiency of backcrossing.

Selection for RP marker alleles can increase greatly the effectiveness of backcross programs by allowing the breeder to 1) select backcross plants that have a higher proportion of RP genome, and 2) select backcross individuals that are better conversions near a mapped donor allele being transferred (i.e., select for less linkage drag). Expressed in practical terms, using genetic markers to assist backcrossing can 1) increase the probability of obtaining a suitable conversion, and 2) decrease the time required to achieve an acceptable recovery.

Issues to consider when planning a marker-assisted backcross program include 1) the time advantage of using markers to assist backcrossing, 2) the number of markers needed, and 3) the number of genotypes to evaluate. In this report, we use results from previous literature, computer simulation, and empirical studies to provide some guidelines.

Table 1. *Expected recovery of recurrent parent (RP) genome during backcrossing, assuming no linkage to the gene being transferred.*

Generation	%RP
F_1	50.0000
BC_1	75.0000
BC_2	87.5000
BC_3	93.7500
BC_4	96.8750
BC_5	98.4375
BC_6	99.2188
BC_7	99.6094

[1]Formerly with Purdue University, West Lafayette, Ind.

Appendix B

Materials and methods

The maize genome was the model for the simulation. The simulated genome contained ten 200-cM chromosomes. Simulation of crossing over was based on a Poisson distribution with a mean of 2.0 ($\lambda = 2$) (Hanson, 1959), which, on average, generated one cross over for every 100-cM length. The simulations reported here assume no interference. Codominant genetic markers were evenly distributed in the genome and sites of the donor gene were randomly assigned to genome locations.

Simulations were conducted with the following parameters:

Number of progeny: 100 or 500.
Backcross generations: BC_1, BC_2, and BC_3.
Number of markers: 20, 40, 80, or 100.
Number selected to form the next BC generation: 1 or 5.

Selection was based on 1) presence of the donor allele and 2) high %RP). %RP was calculated as the average of the (one or five) selected individuals. Values presented are the mean of 50 simulations.

Results

In the computer simulation study, all methods modeled greatly increased the speed of recovering the RP genome compared to the expected recovery with no marker-assisted selection (compare Tables 1 and 2). At least 80 markers were required to recover 99% of the RP genome in just three BC generations (Table 2). Use of at least 80 markers and 500 progeny allowed recovery of 98% RP in just two BC generations. Response to selection was diminished only slightly by spreading the effort over five selections. Using markers, the number of backcross generations needed to convert an inbred is reduced from about seven to three.

By the BC_2 generation, there appears to be no practical advantage to using 500 vs. 100 individuals. If the presence of the donor trait in the backcross individuals can be ascertained before markers are genotyped, then only half the number of individuals indicated in the tables will need to be analyzed.

When a small number of markers are used, they quickly became non-informative; i.e., selection causes the marker loci to became fixed for the RP type before the rest of the genome is fully converted (Table 3; Hospital et al., 1992). This situation was most prominent in the larger populations, where a higher selection intensity placed more selection pressure upon the marker loci. Accordingly, it is of interest to consider how closely the estimation of %RP based on markers reflects the actual genome composition. The combination of estimation of %RP based on fewer markers and subsequent selection tends to bias the estimates upward (compare Tables 2 and 3).

The results from the simulation compare well with real field data. In a typical example, 50 BC_1 plants carrying the gene being transferred were genotyped at 83 polymorphic RFLP loci (note that this corresponds to a population size of 100 unselected plants in Tables 2 and 3). The five best BC_1 recoveries had estimated %RP values of 85.9%, 82.7%, 82.0%, 81.4%, and 81.2%. After evaluating 10 BC_2 plants from each selected BC_1, the best BC_2 recovery had an estimated %RP of 94.6%.

Discussion

The simulations (Table 2; Hospital et al., 1992) and our experience indicate that four markers per 200-cM chromosome is adequate to greatly increase the effectiveness of selection in the BC_1. However, using only four markers per 200 cM will likely make it very difficult to map the location of the gene of interest. Adequate summarization of the data is an important

Table 2. *Percent recurrent parent genome during marker-assisted backcrossing.*

	100 Progeny				500 Progeny			
	No. markers				No. markers			
Generation	20	40	80	100	20	40	80	100
				One selected				
BC_1	84.5	84.5	84.2	88.0	89.9	90.7	90.2	90.5
BC_2	95.0	95.2	95.8	97.2	96.5	97.7	98.5	98.6
BC_3	97.4	97.6	98.9	99.2	97.7	98.3	99.4	99.5
				Five selected				
BC_1	82.9	85.1	84.9	84.7	87.7	88.1	88.9	88.9
BC_2	93.7	95.0	95.8	95.7	95.5	96.8	97.8	97.9
BC_3	97.1	98.3	98.8	98.9	97.3	98.5	99.3	99.3

Table 3. *Estimates of percent recurrent parent genome, based on marker loci.*

	100 Progeny				500 Progeny			
	No. markers				No. markers			
Generation	20	40	80	100	20	40	80	100
				One selected				
BC_2	98.7	97.8	95.6	97.2	100.0	99.1	98.6	98.0
BC_3	100.0	99.8	99.3	99.5	100.0	100.0	99.9	98.2
				Five selected				
BC_2	96.4	96.5	96.2	95.8	100.0	98.5	98.3	98.2
BC_3	99.9	99.8	99.3	99.1	100.0	100.0	99.9	99.8

part of a marker-assisted backcross program. Ideally, the markers used can supply data that can be represented as alleles of loci with known map position. Estimation of %RP, mapping the position of the locus of interest, and graphical display of the results (Young and Tanksley, 1989) are all useful in understanding and controlling the specific backcross experiment being conducted.

It appears that, with the use of genetic markers, the portion of the RP genome that is not linked to the allele being transferred can be recovered quickly and with confidence. The recovery of RP will be slower on the chromosome carrying the gene of interest. A considerable amount of linkage drag is expected to accompany selection for the DP allele in a backcross program. For a locus located in the middle of a 200-cM chromosome, the length of the DP chromosome segment accompanying selection is expected to be 126, 63, and 28 cM in the BC_1, BC_2, and BC_3 generations, respectively (Hanson, 1959; Naveira and Barbadilla, 1992). Our observations support the recommendation of Hospital et al. (1992) that preference be given to the selection for recombinants proximal to the allele of interest, but that selection for recovery of the RP elsewhere in the genome also be considered. This two-stage selection can probably be done quite effectively ad hoc by the breeder once the data is adequately summarized; however, Hospital et al. suggest ways to incorporate the two criteria into a selection index such that each component of selection is assured appropriate weighting.

Use of genetic markers can greatly increase the effectiveness of backcrossing, and they should be used in any serious backcrossing program if resources are available to the breeder.

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Attorney Docket No. 1329

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Brian Douglas Swanson	Date:	March 14, 2003
Serial No.:	09/759,704	Group Art Unit:	1638
Filed:	January 12, 2001	Examiner:	David H. Kruse
For:	"INBRED MAIZE LINE PH6WG"		

Assistant Commissioner for Patents
Washington, D.C. 20231

RULE 132 DECLARATION
OF
DR. STEPHEN SMITH

Sir:

I, Stephen Smith, PhD., do hereby declare and say as follows:

1. I am skilled in the art of the field of the invention. I have a Ph.D. in Biochemical Systematics and Taxonomy of Maize and its Wild Relatives from Birmingham University. I have a M.Sc. in the Conservation and Utilization of Plant Genetic Resources from Birmingham University. I have a Bachelor of Science degree in Plant Sciences from London University. Since 1977 I have been engaged in the development, study and application of molecular markers to genetics, measuring genetic diversity and tracking pedigrees. I commenced this work at North Carolina State University as a post-doctoral research fellow. I have continued my engagement in these studies during my employment by Pioneer Hi-Bred from 1980 until the present. These studies have resulted in numerous scientific articles that have appeared in peer reviewed scientific literature.

2. This declaration is in response to the Examiner's rejection under, 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

3. I have conducted an analysis of Simple Sequence Repeat, SSR, marker data for base inbred PH6WG and ten backcross conversions of PH6WG. The backcross conversions consist of eight backcross conversions for the trait of herbicide resistance, one backcross conversion for the trait of male sterility, and one backcross conversion for the trait of waxy starch.

Appendix C

09/759,704

4. The SSR data for 457 base inbreds and 103 backcross conversion inbreds, including PH6WG and the ten backcross conversions of PH6WG were used in the analysis. The number of SSR markers for each inbred used in the analysis was between 15 and 87 (mean of 82). The analysis was done as specified in the publication by Berry et al. ("Assessing Probability of Ancestry Using Simple Sequence Repeat Profiles: Applications to Maize Hybrids and Inbreds" Genetics 161:813-824, 2002), with modification as described in Berry et al., (2003); Assessing Probability of Ancestry Using SSR Profiles: Application to maize inbred lines and soybean varieties. Genetics (in review), a copy of which is attached hereto.

5. The results of the analysis indicated that through the use of SSR markers PH6WG was identified to be the recurrent parent of each of the ten backcross conversions of PH6WG over all the other inbreds in the data set. The probability associated with the identification of PH6WG as the recurrent parent of each backcross conversion was calculated as 0.99 and higher.

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: March 14th 2003 By: Stephen Smith

Stephen Smith

ASSESSING PROBABILITY OF ANCESTRY USING SIMPLE SEQUENCE REPEAT PROFILES: APPLICATIONS TO MAIZE INBRED LINES AND SOYBEAN VARIETIES

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SHORT RUNNING HEAD:

Probability of ancestry using SSR

KEY WORDS:

Inbred alleles, Parentage, Pedigree, SSR, Bayes' Rule

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ABSTRACT

Determining parentage is a fundamental problem in biology and in applications such as identifying pedigrees. Difficulties inferring parentage derive from extensive inbreeding within the population, whether natural or planned; using an insufficient number of hypervariable loci; and from allele mis-matches caused by mutation or by laboratory errors that generate false exclusions. Many studies of parentage have been limited to comparisons of small numbers of specific parent-progeny triplets. There have been few large-scale surveys of candidates in which there is no prior knowledge of parentage. We present an algorithm that determines the probability of parentage in circumstances where there is no prior knowledge of pedigree and which is robust in the face of missing data and mis-typed data. The focus is parentage of an inbred line having uncertain ancestry. The algorithm is a variation of a previously published hybrid-focused algorithm. We describe the algorithm and demonstrate its performance in determining parentage of 43 inbred varieties of soybean that have been profiled using 236 SSR loci and from seven inbred varieties of maize that were profiled using 70 SSR loci. We include simulations of additional levels of missing and mis-typed data to show the algorithm's utility and flexibility.

The determination of parentage using molecular marker data has been little addressed for situations where there is little or no prior knowledge of parentage, or when large-scale surveys involving numerous candidate parents are required. Consequently, we have recently developed an algorithm and demonstrated its use in determining probability of parentage for hybrids in circumstances where there is no prior knowledge of pedigree and which is robust in the face of missing or mis-typed data (Berry *et al.* 2002). We now present a variation of this algorithm that allows determination of parentage for inbred lines or homozygous varieties.

We describe and evaluate a methodology that quantifies the probability of parentage of homozygous genotypes. Our algorithm takes into account that generations of self-pollination occur after the initial parental cross. The number of generations and the initial parental genotypes are unknown. Each generation of inbreeding reduces the number of heterozygous loci in the progeny by an average of 50%. Thus, each of the inbred progeny individuals resulting from the initial parental cross will have lost approximately half of the parental alleles for loci where the inbred parents were fixed for alternate alleles and which were heterozygous in the F1 generation.

The loss of parental alleles during the inbreeding phase is in contrast to the case of a hybrid progeny. An inbred progeny individual will exhibit a lower level of allelic similarity to either of its inbred parents than a hybrid progeny will to its inbred parents. This loss of some parental alleles during inbreeding might be expected to make an inbred algorithm less robust in the face of missing or mis-typed data compared with the hybrid algorithm that has been previously described (Berry *et al.* 2002). We therefore demonstrate the effectiveness and robustness of the inbred algorithm using examples from two species of cultivated plants. We first tested the

algorithm using varieties of the naturally self-pollinating, inbred crop, soybean [*Glycine max. (L.) Merr.*]. This crop was selected because numerous varieties of soybean with known pedigrees were available to us, many of which are closely related. We also used publicly bred inbreds of maize (*Zea mays L.)* that are of known pedigree. Maize is naturally an outcrossing species but inbred lines are most usually generated for use as parents of commercial hybrids. Inbred lines are generated by making successive generations of self-pollination following the initial bi-parental cross.

MATERIALS AND METHODS

Algorithm: The algorithm is a variation of the hybrid version of Berry *et al.* (2002). Consider an index inbred whose parentage is unknown or in dispute. A database containing possible inbred ancestors is available. The objective is to find the probabilities of closest ancestry for each inbred in the database using genotypic information from a large number of SSRs.

Consider a pair of possible ancestors, inbred *i* and inbred *j*. We calculate the probability that inbreds *i* and *j* are in the index's ancestry, repeating this for all pairs of inbreds in the database. Let $P(i,j|SSRs)$ stand for the posterior probability that *i* and *j* are ancestors of the index given the information from the various SSRs. Let $P(i,j)$ stand for the unconditional (or prior) probability of the same event and let $P(SSRs|i,j)$ be the probability of observing the various SSR results if in fact *i* and *j* are ancestors of the index. Just as in Berry *et al.* (2002), Bayes' rule relates these various probabilities:

$$P(i,j|SSRs) = P(SSRs|i,j)*P(i,j) / \Sigma[P(SSRs|u,v)*P(u,v)],$$

where the sum in the denominator is over all pairs of inbreds in the database, indexed by u and v. We need to calculate $P(SSRs|i,j)$ for each i and j. We will make the "no-prior-information" assumption that $P(i,j)$ is the same for all pairs (i,j). Then $P(u,v)$ is a common multiple in the denominator that cancels with $P(i,j)$ in the numerator:

$$P(i,j|SSRs) = P(SSRs|i,j) / \Sigma P(SSRs|u,v).$$

The problem is to calculate a typical $P(SSRs|i,j)$, the probability of observing the index's SSRs assuming inbreds i and j are both ancestors. The nature of breeding before the self-pollination process is unknown. Since the creation of an inbred proceeds by multiple generations of self-pollination on a hybrid, we label the (unknown) hybrid used to create the (known) index inbred as the intermediate hybrid. When the intermediate hybrid is an immediate descendent of i and j, it receives one of inbred i's alleles and one of inbred j's alleles. When the intermediate hybrid is a second generation descendent of i and j, it receives one allele from each with probability 0.5. And so on. Since degree of ancestry (if any) is unknown, we label the actual probability of passing on one of these alleles to the intermediate hybrid to be p. As in Berry *et al.* (2002) we consider $p = 0.50$ and $p = 0.99$ and here we also consider the intermediate value $p = 0.75$.

When inbreds i and j are ancestors then there are four possibilities: (1) the alleles of both i and j were passed to the intermediate hybrid, (2) i came through but not j, (3) j came through but not i, and (4) neither came through. Assuming independence, these have respective probabilities p^2,

$p(1-p)$, $p(1-p)$, $(1-p)^2$. An allele in the intermediate hybrid's genotype that did not arise from either inbred *i* or inbred *j* is assumed to be selected with probability $1/n$, where n is the total number of alleles at the SSR in question. So far the steps we have described are identical to those for identifying the ancestors of a hybrid described by Berry *et al.* (2002) and, in fact, if the index is heterozygous at an SSR then calculations proceed just as for hybrids. Calculations are substantially different when the index inbred is homozygous, say genotype *aa*. Cases that must be considered are shown in Table 1, where *x* is any allele different from *a* (but not missing). All alleles other than *a* can be grouped because only *a* appears in the index's genotype. For example, *xx* might be *bc* or *bd* or *bb*.

P(SSR|i,j) is the probability of observing the index assuming inbreds *i* and *j* are ancestors. The calculations for SSRs 1 to 6 are shown in Table 2, where the four terms in each case are in order of (1), (2), (3), (4) defined in the previous paragraph. Missing alleles are not considered in the examples above. The number of possibilities is large. Here we consider only the case in which inbred *i* is *aa* and both alleles of inbred *j* are missing. Then

$$P(SSR|i,j) = p^2(1/2+1/2*1/n) + p(1-p)(1/2+1/n*1/2) + p(1-p)(1/n) + (1-p)^2(1/n)$$

Another possibility not considered above is that more than two alleles can be observed for an SSR marker run on individual DNA sample. This can be due to SSR locus duplication, homology due to alloploidy, more than one individual plant being sampled for DNA extraction or cross-contamination. In this case we consider all possible pairings of the observed alleles and calculate using a multiple imputation procedure (Little and Rubin, 1987).

To find the overall $P(SSRs|i,j)$, multiply the individual $P(SSR|i,j)$ over the various SSRs. To determine the probability that any particular inbred, say inbred i, is the closest ancestor of the index, sum $P(SSR|i,v)$ over all inbreds v with $v \neq i$. Call this $P(i|SSRs)$. The maximum of $P(i|SSRs)$ for any inbred i is 1. But since there is one closest ancestor on each side of the family, the sum of $P(i|SSRs)$ over all inbreds i is 2.

SSR data: Soybean DNA was extracted from 490 varieties, all of which were bred in, and are adapted to, the United States. Plant material for DNA extraction was sampled from six plants of each variety. Most of the varieties are proprietary products of Pioneer Hi-Bred International. Several (non-patented) commercial varieties from other breeding companies and some important publicly bred varieties were also included. Procedures for obtaining SSR data from soybean were identical to those described for maize by Berry *et al.* (2002) apart from the following modifications: PCR products with different size ranges and labeled with different fluorochromes were pooled and diluted 1:9 with capillary electrophoresis buffer (Applied Biosystems) then 1:4 with dH20. 1.5ul of pooled DNA were added to 10ul formamide containing the molecular weight size standard 400HD ROX (Applied Biosystems, ROX = 6-carboxy-X-rhodamine). Fragment separation was performed using capillary electrophoresis on an ABI3700 platform (Applied Biosystems), with an injection time of 10 sec at 10,000 V and a run time of 4,000 sec at 7,500 V. Forty-three soybean varieties that had both of their parent varieties also included in the dataset were assigned as index varieties. One to two and occasionally three grandparent varieties of several of the index varieties were also included in the dataset. These varieties collectively

represent a broad array of diversity of soybean germplasm that is currently grown in the United States.

Two hundred and thirty-six publicly available soybean SSR markers (http://soybase.agron.iastate.edu/) were used to demonstrate and evaluate the algorithm. These SSR markers were selected following initial screens on a subset of 24 soybean varieties in which they were tested for amplification and the ability to detect polymorphism. The 236 markers gave good genome coverage and collectively mapped across each of the chromosomal linkage groups of soybean.

All allele scores were made without knowing the identities of the soybean genotypes.

Maize SSR data using 70 loci were previously reported by Senior *et al.* (1998) and were obtained directly from the first author. This publication (Senior *et al.* 1998) cites an array of 94 historically important publicly bred lines that have well known and well established pedigrees. This array of public inbreds includes seven inbreds (A632, A634, Mo17, Pa91, Va35, Va99 and W64A) that each have SSR profiles for their parental lines included in the same dataset. Three of these inbreds were developed from a breeding cross of two unrelated parents. These are: Mo17 which was bred from the cross of C.I. 187-2 x C103; Va99, which was bred from the cross Oh07B x Pa91; and W64A which was bred from the cross of WF9 x C.I. 187-2. Other inbred progeny had more complex pedigrees. One inbred (Va35) was bred from the cross C103 x T8 following an additional cross of T8 as the recurrent parent. Two inbreds (A632 and A634) were bred from the cross Mt42 x B14 following additional crosses of B14 as the recurrent parent.

Pa91 was bred from a complex cross involving four inbreds (WF9 x Oh40B) and (38-11 x L317). These seven progeny inbreds therefore provided an index set of maize inbreds for evaluation of the inbred algorithm.

RESULTS

Data quality: The soybean SSR data that were used to evaluate the algorithm had a mean of 5.5% (range 0-19% loci) missing data per variety. For parent-progeny triplets, there was a mean of 1.1% loci (range 0-5%) where a progeny profile was scored for an allele that was not represented by either of the seed sources that represented the parents. The maize SSR data had a mean of 0.7% missing data (only three genotypes had missing data; these were at elevated levels of 5%, 9%, and 36%). A mean of 6.4% parent/progeny triplets (range 4-7%) had SSR progeny profiles that did not share an allele with either of the seed sources that were available to represent the original parental genotypes.

Probability of ancestry applied to soybean data: Figures 1 and 2 present the probabilities of closest ancestry of the top ranking varieties for each of 43 soybean varieties using data from 236 marker loci at $p = 0.50$ (Fig 1) and at $p = 0.99$ (Fig 2).

When the algorithm was used at $p = 0.5$ with data from all 236 loci (Fig 1), then 24/43 (56%) of index varieties had both parents correctly identified in the top two ranked positions, 12/43 (28%) had one parent correctly placed in one of the top two positions, and 7/43 (16%) had none of the actual parents assigned into the top two ranked positions. Thus, when $p = 0.5$ was used, 60/86

(70%) of actual parental varieties were correctly ranked in the top two positions and 26/86 (30%) were incorrectly placed in lower positions.

When the algorithm was used at $p = 0.75$ with data from all 236 loci (data not shown), 28/43 (65%) of index varieties had both parents correctly identified in the top two ranked positions, 11/43 (26%) had one parent correctly placed in one of the top two positions, and 4/43 (9%) had none of the actual parents assigned into the top two ranked positions. Therefore, when $p = 0.75$ was used, 67/86 (78%) of the actual parental varieties were correctly ranked in the top two positions and 19/86 (22%) were incorrectly placed in lower positions.

When the algorithm was used at $p = 0.99$ with data from all 236 loci (Fig 2), then 33/43 (77%) of actual parental varieties were correctly ranked in the top two positions and 10/86 (23%) had one parent correctly placed; all index varieties had at least one parent ranked in the top two positions when the algorithm was used at $p = 0.99$. With p used at 0.99 then 76/86 (88%) of actual parental varieties were correctly assigned; 10/86 (12%) were incorrectly assigned.

Table 3 presents the rankings, probabilities, and pedigrees of varieties that were incorrectly assigned above a true parent. The largest pedigree class (41% of cases where a non-parent ranked above a true parent) of non-parents ranking higher than parents was for varieties that are derivatives of the parent that was misplaced at a lower ranking. The equal second largest classes (each representing 14% of the cases) were for varieties that were (a) full sibs of the true but misplaced parent and (b) full sibs of a grandparent of the variety for which the pedigree was being tested. Other categories (percent of cases in parentheses) were: multiple backcross versions

of the misplaced parent (7%), a derivative of the variety or which the pedigree was being tested (7%), a half-sib of the true but lower ranked parent (7%), a full sib of the variety for which the pedigree was being tested (3%), and a half-sib of the variety for which the pedigree was being tested (3%). Insufficiently detailed pedigree information is available to categorize one variety (3% of cases) that ranked above the true parent

Robustness: The quality of soybean SSR data as received from the laboratory, in terms of missing data and apparently non-Mendelian parent-progeny triplets, have already been presented. Taking these data as an initial starting point, additional levels of missing and mis-typed data were created by simulations and used to explore robustness of the algorithm.

SSR data for five index soybean varieties were used to determine the robustness of the algorithm. Subsets of data were created that included parameters of reduced numbers of loci, additional levels of missing data, additional levels of mis-typed data, and various combinations of these parameters. Simulated levels of missing and mis-typed data were created with a first pass creating missing data, followed by a second pass creating mis-typed data. Therefore, for example, the maximum level of cumulative error from simulated missing and mis-typed data was from 36 to 40%. Five varieties were chosen to represent a range of diversity in respect of both pedigree and SSR profiles. Four varieties had no parents or grandparents in common and one pair of varieties was related by a common parent. All varieties had parents ranked in the top two positions when the algorithm was run at $p = 0.75$ and $p = 0.99$. This selection of varieties therefore provides a means to establish lower boundaries for both the quantity and quality of SSR data that are required to avoid aberrant results.

Table 4 presents the probability of ancestry of the top five ranked varieties for each of five selected soybean index varieties (93B11, A7986, P9443, S38T8 and Young) when the algorithm is run using different numbers of SSR marker loci (50, 100, 150 and 236) at each of two levels of p (0.5 and 0.99). Using $p = 0.5$, the lowest percentage of parents (60%) that were correctly ranked into the top two positions corresponded to using only 50 SSR. Increasing the number of loci to 100 or 150 or 236 increased the ability to identify the actual parents to about 90%. When p was used at a level of 0.99 all parents were correctly ranked into the top two positions for each of the five varieties when data from as few as 50 SSR loci were used.

Table 5 summarizes other aspects of robustness. Namely, we simulated additional levels of missing, mis-typed and missing plus mis-typed data, beyond those that were inherent in the data as provided by the laboratory. When p was used at a level of 0.5, robustness was generally maintained up to an additional level of 20% simulated missing data, so long as data from 100 or more loci were used. Similarly, robustness was maintained for up to 20% additional mis-typed data so long as data from 100 or more loci were used. Likewise, robustness was maintained with up to 18 to 20% additional levels of data error including both missing and mis-typed data, so long as data from 150 or more loci were used. Using data for all 236 loci provided a higher level of robustness, but even then robustness collapsed when 36 to 40% cumulative additional error from missing and mistyped data were simulated into the analysis. The overall level of correct assignation of parent varieties was higher when p was used at a level of 0.99. All parents then were correctly identified, even when data from only 50 loci were used up to an additional level of 10% missing data. When data from 100 or more loci were used then all parents were correctly

identified with up to 20% additional missing data. Robustness started to decline when the algorithm was applied with 10% additional mis-typed data when data from 150 or fewer SSR loci were used. However, robustness was maintained for up to 20% additional mis-typed data when data from 236 SSR loci were used. When additional levels of both incorrect data were applied then robustness was maintained at levels of up to 10% missing plus 10% mis-typed data so long as data from at least 150 SSR loci were used. Robustness was compromised when additional simulations of 20% missing plus 20% mis-typed data were applied even when data from all 236 SSR loci were used.

We then investigated the relationships of varieties to the index genotype whose pedigree was under examination by rerunning the analysis after both parents of the index genotype had been removed from the analysis. Fifteen varieties that had two or more of their grandparents profiled in the dataset were used for this examination. After removing parents, direct pedigreed derivatives of the index genotype ranked first for P9583, in the first three places for A2943 and in the first six places for P9561. Once all parents and derivatives of the index genotype had been removed from the analysis then the following results were obtained. Predominant classes of varieties ranking in the top five positions were (percent of cases in parentheses): derivatives of the grandparent of the index variety (32%), grandparents of the index variety (16%), derivatives of the parents of the index variety (16%), and half-sibs of the index variety (13%). Grandparents ranked among the first four positions for 10 varieties and were in the first place for five varieties. Great-grandparents ranked within the first seven places for three varieties, and a great-great-grandparent ranked in eighth place for one variety. Other varieties that ranked in the first place were usually closely related to the variety whose pedigree was under examination; full-sibs and

half-sibs were the predominant classes of relatives other than grandparents in the first ranking position after parents and direct derivatives of the variety under examination had been removed.

Probability of ancestry applied to corn data: The seven index inbreds of maize were selected because they represented all of the inbred lines published upon by Senior *et al.* (1998) that had all of their inbred parents also included in the SSR dataset. All of the inbred lines published by Senior *et al.* (1998) have well known and well established pedigrees that are fully provided by those authors.

Table 6 presents probabilities of ancestry for the top five ranked inbreds for each of the seven index inbred lines at two levels of p (0.5 and 0.99). For the three progeny that were bred from single crosses without any subsequent use of one of the parents to make a recurrent cross prior to inbreeding (Mo17, Va99, and W64A) then use of the algorithm at either $p = 0.5$ or at $p = 0.99$ resulted in the parental inbreds being ranked in first and second positions. Use of the algorithm at $p = 0.99$ provided greater discrimination for probabilities of ancestry that were assigned to actual parents compared to highest ranking non-parents. This was most noticeable for the case of inbred Va99 which had a relatively low value when used at $p = 0.5$ for parent 2 (0.5221) compared to parent 1 (0.9999) or to the third ranked inbred (and non-parent), Va22 (0.4252). In contrast, when the program was run at $p = 0.99$ then parent1 and parent2 for Va99 had probabilities of 1 and 0.9855, respectively, with the probability of the third ranked inbred being 0.0131.

For each of the three progeny inbreds that originated from breeding schemes that involved one or more additional crosses of one of their parents, using the algorithm at $p = 0.5$ resulted in

placement of the respective recurrent parent with the highest probability of ancestry. Raising the level of p to 0.99 resulted in both parents (B14 = recurrent parent and MT42 the non-recurrent parent) of the index inbred A632 being ranked in the top two places. Using this level of p also caused a higher ranking (third position) for the non-recurrent parent (MT42) of index inbred A634. Use of p at 0.99 did not cause the non-recurrent parent (C103) of index inbred (Va35) to rank into the top five places.

For the index inbred (Pa91) that was bred from a more complex cross involving four inbred lines, the use of p at 0.5 or at 0.99 resulted in the two parents (WF9 and Oh40B) being ranked in second and third places; highest ranked was inbred Va99 (Va99 is derived from the index inbred Pa91). Neither of the two remaining parents of Pa91 ranked in the top five places.

DISCUSSION

The current widely used North American soybean varieties are founded upon a relatively narrow genetic base of diversity. Gizlice *et al.* (1994) document that the U. S. soybean germplasm base is founded upon 20 plant introductions and that subsequent breeding has made repeated use of related parents. Molecular marker comparisons of elite U. S. soybean varieties compared to a sample of exotic varieties reinforce the conclusion that there is a relative paucity of genetic variation in U. S. soybeans. Narvel *et al.* (2000) have shown that the number of alleles detected among the exotics was 30% greater than among U. S. varieties. Thompson and Nelson (1998) report that very little exotic germplasm has been incorporated into the existing U. S. soybean germplasm base. Examining all pairs of pedigree relationships among the 490 soybean varieties

employed in this study showed that approximately 50% of pairwise relationships are related at the level of half-sib or closer; approximately 10% of pairs are related at the level of full-sib or closer. This set of soybean varieties therefore provides the basis for an extremely rigorous evaluation of the ability of SSR data to distinguish between varieties and of this algorithm to identify pedigrees. Pedigree breeding, including the use of related parents, is also commonly applied in the breeding of maize inbred lines. The set of maize inbreds used here thus also provides a meaningful evaluation of the marker data to discriminate among inbred lines and of the joint ability of the algorithm and of the marker data to allow a determination of inbred pedigrees.

Use of the algorithm at $p = 0.99$ rather than at a lower level improved performance in terms of the percentage of correct assignations of parents and provided a greater statistical differential for probabilities for parents in comparison to the highest ranking non-parents. Use of the algorithm at $p = 0.99$ is more appropriate when it is known that the actual parents of the variety under examination are included among the set of index varieties. If it is not known that the parents are included in the index set then use of the algorithm at $p = 0.5$ is more justified (Berry *et al.* 2002). For the soybean varieties, when p was used at 0.99, then 77% of all varieties that were queried for their parents had both parents correctly identified. Eight-eight percent of soybean parents were correctly identified across 43 index varieties that were queried for their parents. All varieties (with the possible exception of one variety where detailed pedigree information was not available) that ranked above true parents were related either to the mis-ranked parent or to the variety that was being queried for its pedigree. Our previous report of the use of an algorithm to determine hybrid pedigrees (Berry *et al.* 2002) showed a higher level of correct parental

determinations at $p = 0.99$. Many of these soybean varieties have a high degree of pedigree relatedness. However, many of the maize inbred lines that were used in the previously reported study (Berry *et al.* 2002) were also highly related. It is, however, likely to be inherently more challenging to correctly identify parents following cycles of inbreeding because half of the alleles that are segregating in the first generation following the initial breeding cross will be subsequently lost as recurring cycles of self-fertilization occur. Thus, many of the alleles that are present in a hybrid, and which can therefore contribute to the identification of its pedigree, do not remain present in an inbred homozygous progeny.

We examined the pedigrees of soybean index varieties when both parents of the index had been removed from the set of candidate varieties. Direct pedigree descendents with the index variety as one parent then usually ranked higher than other varieties, including varieties that were grandparents or sister varieties of the index variety. When all parents and direct derivatives of the index variety were excluded from the analysis then the predominant classes of varieties ranking in the top five positions were derivatives of the grandparent of the index variety (32%), grandparents of the index variety (16%), derivatives of the parents of the index variety (16%), and half-sibs of the index variety (13%). The SSR data that were available to us did not allow a thorough or very precise assessment of how varieties with different degrees of relatedness would rank as members of the pedigree in the event that the true parents were not present in the database. Nonetheless, when parents were excluded from the analysis then varieties that were very closely related to the index variety ranked highest. Direct descendents dependent for their pedigree upon the index variety, if present, tended to rise above varieties included within other classes of pedigree relationship to the index variety. When varieties directly descended by

pedigree from the index variety were also excluded then a grandparent ranked into first position for 33% of the varieties that were examined. Direct pedigree derivatives of one or more of the parents of the index variety had an equal level of occurrence when parents and derivatives of the index variety were excluded. Further investigations of the identification of grandparents will require a dataset including all grandparents of each index variety and will also require a revised algorithm to take account of pedigree contributions from four varieties as opposed to pairs of varieties which forms the basis of the current inbred algorithm.

For the maize inbred line pedigrees, use of the algorithm either at $p = 0.5$ or at $p = 0.99$ resulted in the correct identification of both parents in all cases where the breeding scheme was an initial cross of two parental lines followed by subsequent cycles of inbreeding (i.e. for the inbreds Mo17, Va99 and W64A). The relatively high level of robustness for results with maize inbreds at $p = 0.5$, in contrast to the results obtained from analyzing soybean data (where 56% of varieties had both parents correctly identified when p = 0.5 was used) could be accounted for by the smaller sample size of maize inbreds and by the lower degree of mean pedigree relatedness amongst this selection of inbred lines in comparison to the soybean varieties. Thus while several inbred lines in this set are closely related, there remain many inbreds that have little or no pedigree relationship (Senior *et al.* 1998).

The inbred algorithm correctly identified both parents of the three maize index inbreds that had been bred from bi-parental crosses that involved equal contributions (by pedigree) from both parents. For the three bi-parental crosses that involved subsequent additional crosses of the recurrent parent (and thus significantly biased contributions by pedigree to the index variety

from the recurrent parent) then use of the algorithm correctly identified each of the recurrent parents. The algorithm was unable to identify the non-recurrent parent in most cases, but this result would be expected because one backcross reduces the expected pedigree contribution of the non-recurrent inbred to 25%. More generations of backcrossing using the recurrent parent then further reduce the expected pedigree contribution of the non-recurrent parent by half at each generation (successively to 12.5%, 6.25%, 3.125%) with the pedigree contribution of the recurrent parent rising accordingly. Since several inbred lines of maize are related by pedigree then it is not surprising that the level of pedigree or SSR similarity of a non-recurrent parent to the index progeny can fall below other inbred lines that are related to the index variety. The algorithm was not able to preferentially identify parents of the inbred line Pa91, which was bred from a complex breeding scheme involving four parents with equal contributions by pedigree. A more suitable algorithm is needed to take account of four way crosses. However, such a need is primarily academic because most breeding crosses in commercial maize breeding, and indeed for most crops, are bi-parental.

These soybean data had a mean of 5.5% missing data per variety and a mean of 1.1% loci where a progeny was scored with an allele that was not also scored in either or both parents. Such apparent non-Mendelian or exclusionary profiles can be due to pollen contamination during inbreeding, cross contamination in the field or laboratory, scoring errors in the laboratory (e.g. scoring +A, predominant stuttering, spectral pull-up, secondary binding sites or polymer spikes), or incorrect pedigrees. Another source of apparent exclusion is through the use of a seed source as a parent that is still heterogeneous due to inbreeding being incomplete. Cycles of inbreeding then continue so that when those seed sources are used in the future as sources for SSR profiling

to represent the parental genotype they will have lost alleles due to inbreeding that have already been passed on to a progeny. Alternately, residual heterozygosity within seed sources can result in low frequencies of heterozygotes or off-type segregants which may, by chance, be sampled in the progeny, but not sampled in the parent. In this study we sampled six plants to represent the variety which may be insufficient to capture alleles existing at low frequencies within the seed source. And even if the allele was sampled, it may not have been detected following PCR amplification due to predominance of the most frequent allele and allelic competition effects. Hall (2002) has also reported the occurrence of apparent non-parental SSR alleles. Mutation can also affect SSR profiles. Vigouroux *et al.* (2002) have estimated mutation rates of 7.7×10^{-4} per generation for dinucleotide SSRs and an upper 95% confidence limit of 5.1×10^{-5} for SSRs with longer repeat units. A level of error or discrepancy in expected SSR profiles are thus inevitable for some, if not all crop plants. We therefore evaluated the robustness of the algorithm and dataset by rerunning the algorithm using datasets that were simulated to have up to 20% additional levels of missing plus 20% mis-typed data beyond the level that was received from the laboratory. The algorithm maintained its initial level of robustness with up to an additional level of 10% both missing and mis-typed data, provided data from at least 100 SSR loci were used. Fewer loci (60) were capable of retaining this degree of robustness in the evaluation of the hybrid pedigree algorithm using maize hybrids (Berry *et al.* 2002). The loss of parental alleles that occurs during the inbreeding process, in contrast to their retention in a hybrid progeny compared to its parents, probably underlies the need to use data from a greater number of loci to maintain robustness for the inbred algorithm as compared to the hybrid algorithm.

It was anticipated that determination of pedigrees following cycles of inbreeding might be more challenging to accomplish than to determine pedigrees of hybrids where the total nuclear genetic contributions of both parents are preserved. Nonetheless, these results show that the algorithm can be used effectively to identifying parents of inbred genotypes. Nearly 90% of soybean parents were identified. This is a set of genotypes which, due to the relatively narrow founder base and subsequent cycles of development through the use of related crosses, provides an extremely rigorous test of the algorithm and of the discriminatory power of the marker data. Supplementary data also show the capability of the algorithm to identify parents of maize inbreds that have been developed in a pedigree system using two parents. Use of this algorithm with currently available codominantly expressed molecular marker data has also been shown to have practical feasibility because of the high degree of robustness that is evident and which extends well beyond the realm of aberrant or unexpected marker data that is encountered. These types of error or unexpected marker data can include laboratory error, sampling effects or the use of different seed sources for the actual parental source compared to a more inbred source that becomes available later to represent the parental genotype. This algorithm has application in a number of fields, including conservation biology, population genetics, and to assist in the protection of intellectual property rights.

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Table 1. Calculations of ancestry for homozygous index inbreds: Cases that must be considered for example of genotype *aa*.

SSR	Index	Inbred *i*	Inbred *j*
1	*aa*	*aa*	*Aa*
2	*aa*	*aa*	*Ax*
3	*aa*	*aa*	*Xx*
4	*aa*	*ax*	*Ax*
5	*aa*	*ax*	*Xx*
6	*aa*	*xx*	*Xx*

x is any allele different from *a*, but not missing

Table 2. Probability of observing the index [$P(SSR|i,j)$] assuming inbreds i and j are ancestors: Calculations for SSRs 1 to 6.

SSR	$P(SSR\|i,j)$
1	$p^2(4/4) + p(1-p)(1/2+1/n*1/2) + p(1-p)(1/2+1/n*1/2) + (1-p)^2(1/n)$
2	$p^2(3/4) + p(1-p)(1/2+1/n*1/2) + p(1-p)(1/2*1/2+1/n*1/2) + (1-p)^2(1/n)$
3	$p^2(2/4) + p(1-p)(1/2+1/n*1/2) + p(1-p)(1/n*1/2) + (1-p)^2(1/n)$
4	$p^2(2/4) + p(1-p)(1/2*1/2+1/n*1/2) + p(1-p)(1/2*1/2 + 1/n*1/2) + (1-p)^2(1/n)$
5	$p^2(1/4) + p(1-p)(1/2*1/2+1/n*1/2) + p(1-p)(1/n*1/2) + (1-p)^2(1/n)$
6	$p^2(0/4) + p(1-p)(1/n*1/2) + p(1-p)(1/n*1/2) + (1-p)^2(1/n)$

The four terms in each case are in order of the four possibilities when inbreds i and j are ancestors: (1) the alleles of both i and j were passed to the intermediate hybrid, (2) i came through but not j, (3) j came through but not i, and (4) neither came through. Missing alleles are not considered.

Table 3. Probabilities of ancestry and pedigree relationships for soybean varieties where both parents did not rank above non-parents.

Case no.	Index variety	Rank	Possible ancestor	Probability
1	*95B97*	1	Parent 2	1
		2	Full sib of parent 1	0.5822
		3	Parent 1	0.4124
2	*A2943*	1	Parent 1	0.9977
		2	Multiple backcross of parent 2	0.7999
		3	Parent 2	0.1999
3	*A4595*	1	Parent 1	1
		2	Derivative of parent 2	0.9956
		3	Multiple backcross of parent 2	0.0034
		4	Derivative of Parent 2	0.0006
		5	Half sib of A4595	0.0004
		6	Parent 2	0.0001
4	*Hark*	1	Parent 1	1
		2	Derivative of parent 2	1
		3	Derivative of parent 2	2.1E-09
		4	Derivative of parent 2	1.4E-09
		5	Derivative of Hark	3.1E-10
		6	Derivative of parent 2	1.1E-13
		7	unknown	3.8E-15
		8	Derivative of parent 2	4.6E-17
		9	Derivative of parent 2	4.7E-21
		10	Parent 2	2.7E-21
5	*Kent*	1	Parent 2	1
		2	Derivative of parent 1	0.9990
		3	Derivative of parent 1	0.0011
		4	Parent 1	3.0E-04
6	*P9583*	1	Parent 1	1
		2	Full sib of P9583	0.8801
		3	Parent 2	0.1199
7	*P9641*	1	Parent 2	1
		2	Derivative of P9641	1
		3	Parent 1	3.7E-06
8	*S30J2*	1	Parent 1	1
		2	Derivative of parent 2	0.9321

		3	Parent 2	0.0679
9	*YB30K01*	1	Parent 2	1
		2	Half sib of parent 1	1
		3	Full sib of parent 2	7.9E-09
		4	Half sib of parent 2	3.3E-09
		5	Full sib of grandparent	1.2E-10
		6	Derivative of parent 1	3.0E-11
		7	Full sib of parent 2	2.0E-11
		8	Full sib of grandparent	8.7E-12
		9	Parent 1	1.1E-12
10	*YB41Q01*	1	Parent 2	1
		2	Full sib of parent 1	1
		3	Full sib of grandparent	7.3E-05
		4	Full sib of grandparent	4.1E-09
		5	Parent 1	9.1E-10

Results for 33 (77%) varieties where both parents were ranked first and second are not included in this table (see Figures 1 and 2).

Table 4. Probability of ancestry for five individual soybean varieties using SSR data obtained from different numbers of loci (50, 100, 150, 236).

	L50		L100		L150		L236	
Inbred	Possible ancestor	Prob	Possible ancestor	Prob	Possible ancestor	Prob	Possible ancestor	Prob
P=0.5								
93B11	*XB31C*	0.9461	*XB31C*	1	*XB31C*	1	*XB31C*	1
	A3415	0.8006	*A3415*	0.9362	*A3415*	0.9146	*A3415*	0.9954
	XB38A01	0.0256	*WILLIAMS*	0.0429	*WILLIAMS*	0.0809	*WILLIAMS*	0.0046
	P9271	0.0251	*A3242*	0.0155	*YB30L01*	0.0034	*A3242*	0
	YB30L01	0.0232	*YB30L01*	0.0015	*A3242*	0.0006	*DOUGLAS*	0
A7986	*COOK*	0.7748	*BRAXTON*	0.9725	*BRAXTON*	1	*BRAXTON*	1
	XB63D00	0.2841	*YOUNG*	0.5302	*YOUNG*	0.8910	*YOUNG*	0.9929
	S6262	0.1826	*COOK*	0.3872	*P9641*	0.0404	*XB63D00*	0.0071
	YOUNG	0.1755	*XB63D00*	0.0496	*XB63D00*	0.0254	*96B32*	0
	BRAXTON	0.1065	*P9641*	0.0328	*COOK*	0.0245	*P9641*	0
P9443	*DOUGLAS*	0.8086	*A3415*	0.5557	*FAYETTE*	0.8760	*FAYETTE*	0.9885
	A3415	0.7629	*FAYETTE*	0.4957	*A3415*	0.7034	*DOUGLAS*	0.8847
	WILLIAMS	0.0887	*DOUGLAS*	0.4855	*CX399*	0.1671	*A3415*	0.0846
	YALE	0.0501	*CX260C*	0.2032	*CX260C*	0.1273	*WILLIAMS*	0.0348
	P9394	0.0411	*WILLIAMS*	0.1608	*WILLIAMS*	0.0948	*CX399*	0.0062
S38T8	*S3535*	0.8711	*S3535*	0.9993	*S3535*	1	*S3535*	1
	S4644	0.4543	*S4644*	0.9988	*S4644*	1	*S4644*	1
	YB44R01	0.2762	*YB40M01*	0.0012	*YB37Y00*	0	*A4268*	0
	YB40M01	0.1087	*YB44R01*	0.0004	*93B65*	0	*YB44R01*	0
	YB44Q01	0.0325	*YB37Y00*	0.0001	*A4268*	0	*YB37Y00*	0
YOUNG	*DAVIS*	0.6589	*DAVIS*	0.6551	*DAVIS*	0.6324	*DAVIS*	0.9752
	XB63D00	0.4942	*ESSEX*	0.5979	*P9641*	0.5524	*P9641*	0.5397
	96B32	0.3122	*P9641*	0.3409	*COOK*	0.3231	*ESSEX*	0.3273

	COOK	0.0707	*COOK*	0.1692	*ESSEX*	0.2817	*96B32*	0.1299
	OGDEN	0.0606	*96B32*	0.1315	*96B32*	0.1933	*COOK*	0.0235
p=0.99								
93B11	*XB31C*	1	*XB31C*	1	*XB31C*	1	*XB31C*	1
	A3415	0.9999	*A3415*	0.9999	*A3415*	1	*A3415*	1
	A3242	0.0001	*A3242*	0.0001	*P9443*	0	*WILLIAMS*	0
	P9443	0	*P9443*	0	*A3242*	0	*A3242*	0
	WILLIAMS	0	*WILLIAMS*	0	*WILLIAMS*	0	*FAYETTE*	0
A7986	*BRAXTON*	1	*BRAXTON*	1	*BRAXTON*	1	*BRAXTON*	1
	YOUNG	0.9903	*YOUNG*	0.9903	*YOUNG*	0.9987	*YOUNG*	1
	P9641	0.0092	*P9641*	0.0092	*96B32*	0.0012	*XB63D00*	0
	96B32	0.0005	*96B32*	0.0005	*P9641*	0.0002	*96B32*	0
	DAVIS	0	*DAVIS*	0	*DAVIS*	0	*P9641*	0
P9443	*DOUGLAS*	0.9998	*DOUGLAS*	0.9999	*FAYETTE*	0.9995	*DOUGLAS*	1
	FAYETTE	0.7010	*FAYETTE*	0.7011	*DOUGLAS*	0.9993	*FAYETTE*	1
	CX260C	0.2345	*CX260C*	0.2345	*CX399*	0.0006	*CX260C*	0
	A3415	0.0644	*A3415*	0.0643	*A3415*	0.0005	*CX399*	0
	S3941	0.0001	*AP3330*	0.0001	*P9394*	0.0001	*A3415*	0
S38T8	*S3535*	1	*S3535*	1	*S3535*	1	*S3535*	1
	S4644	1	*S4644*	1	*S4644*	1	*S4644*	1
	YB40M01	0	*YB40M01*	0	*93B67*	0	*A4268*	0
	YB44R01	0	*A5979*	0	*ST3780*	0	*YB54J00*	0
	93B67	0	*YB44R01*	0	*YB37Y00*	0	*YB44R01*	0
YOUNG	*DAVIS*	1	*DAVIS*	1	*DAVIS*	1	*DAVIS*	1
	ESSEX	1	*ESSEX*	1	*ESSEX*	1	*ESSEX*	1
	P9641	0	*P9641*	0	*COOK*	0	*S4240*	0

Attorney Docket No. 1329

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Brian Douglas Swanson	Date:	March 14, 2003
Serial No.:	09/759,704	Group Art Unit:	1638
Filed:	January 12, 2001	Examiner:	David H. Kruse
For:	"INBRED MAIZE LINE PH6WG"		

Assistant Commissioner for Patents
Washington, D.C. 20231

RULE 132 DECLARATION
OF
DR. DINAKAR BHATTRAMAKKI

Sir:

I, Dinakar Bhattramakki, Ph.D., do hereby declare and say as follows:

1. I am skilled in the art of the field of the invention. I have a Ph.D. in Plant Molecular Genetics from the University of Illinois at Urbana-Champaign. I have a Bachelor of Science degree in Agricultural Sciences from the University of Agricultural Sciences, Bangalore, India. Since 1997 I have been engaged in the analysis of molecular markers for plants. I have supervised the Molecular Marker Applications lab at Pioneer Hi-Bred International, Inc. from January 2002 until the present.

2. I am familiar with the methods used in the analysis of Simple Sequence Repeat, SSR, marker data for inbred PH6WG conducted at Pioneer Hi-Bred International, Inc. The analysis of the SSR profile of inbred PH6WG may be accomplished without any undue experimentation. The SSR profile for inbred PH6WG is attached hereto.

3. Means of performing this genetic marker profile are well known in the art. SSRs are genetic markers based on polymorphisms in nucleotide sequences. The PCR™ detection of SSRs is accomplished by using two oligonucleotide primers flanking the polymorphic segment of DNA. Amplification is accomplished through

Appendix D

repeated cycles of heat denaturation of the DNA followed by annealing of the primers to their complementary sequences at low temperatures, and extension of the annealed primers with DNA polymerase.

4. Markers are scored following amplification and gel electrophoresis of the amplification products. Scoring of marker genotype is based on the size or weight of the amplified fragment. While variation in the primer used or in laboratory procedures can affect the reported marker score, relative values remain constant regardless of the specific primer or laboratory used.

5. Primers that may be used to identify the SSR markers reported herein are publicly available and may be found in the Maize DB on the World Wide Web at agron.missouri.edu/maps.html (sponsored by the University of Missouri), in Sharopova et al. (Plant Mol. Biol. 48(5-6):463-481) and/or in Lee et al (Plant Mol. Biol. 48(5-6); 453-461). Markers shown for PH6WG are the publicly available markers in the sources listed above for which PH6WG was tested and shown to be homozygous.

6. Map information is provided by bin number as reported in the Maize DB. The bin number digits to the left of decimal point typically represent the chromosome on which such marker is located, and the digits to the right of the decimal typically represent the location on such chromosome.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: By:_____

Dinakar Bhattramakki

09/759,704

Public Name of Marker	bin #	PH6WG base pairs
phi427913	1.01	129.38
bnlg1014	1.01	124.16
phi056	1.01	255.30
bnlg1007	1.02	134.45
bnlg1083	1.02	221.86
bnlg1127	1.02	112.28
bnlg1953	1.02	247.56
bnlg1429	1.02	204.64
bnlg1627	1.02	202.70
bnlg439	1.03	226.98
phi109275	1.03	142.91
phi339017	1.03	155.08
bnlg1203	1.03	300.23
bnlg1484	1.03	157.83
dupssr26	1.04	124.50
bnlg2086	1.04	217.79
bnlg2238	1.04	222.47
bnlg1832	1.05	215.70
bnlg1886	1.05	141.48
bnlg1057	1.06	247.00
bnlg1041	1.06	194.58
bnlg1615	1.06	211.96
bnlg1556	1.07	201.94
phi323065	1.08	329.53
phi335539	1.08	88.58
phi423298	1.08	133.68
bnlg2228	1.08	246.31
phi002	1.08	73.53
bnlg1331	1.09	141.84
phi011	1.09	226.98
phi308707	1.10	131.11
phi227562	1.11	322.45
phi265454	1.11	234.33
phi064	1.11	94.65
phi402893	2.00	218.34
phi96100	2.01	284.43
bnlg1017	2.02	195.57
bnlg2277	2.02	308.44
bnlg1327	2.02	300.60
bnlg1064	2.03	188.18
bnlg1537	2.03	121.81
bnlg1018	2.04	126.05
bnlg1036	2.06	182.95
phi328189	2.08	115.47
phi427434	2.08	121.46
phi435417	2.08	217.99

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Public Name of Marker	bin #	PH6WG base pairs
bnlg1141	2.08	152.92
bnlg1258	2.08	245.05
phi127	2.08	123.97
bnlg1520	2.09	285.85
phi101049	2.10	234.30
phi453121	3.00	223.43
phi104127	3.01	162.19
phi404206	3.01	303.14
phi193225	3.02	136.71
phi374118	3.02	225.95
bnlg1144	3.02	134.68
bnlg1523	3.03	264.89
bnlg1035	3.05	101.06
phi053	3.05	166.74
bnlg1160	3.06	220.49
bnlg1951	3.06	121.12
bnlg2241	3.06	142.63
bnlg1496	3.09	186.02
phi072	4.00	141.56
phi213984	4.01	284.60
phi295450	4.01	184.83
bnlg1162	4.03	92.86
phi021	4.03	92.44
phi308090	4.04	218.64
phi096	4.04	234.58
phi438301	4.05	211.81
bnlg1159	4.05	147.81
bnlg1755	4.05	214.54
bnlg1937	4.05	227.36
bnlg1265	4.05	194.44
phi079	4.05	187.36
bnlg1137	4.06	134.13
umc2038	4.07	135.37
bnlg1189	4.07	140.17
bnlg1784	4.07	233.02
bnlg2244	4.08	199.58
bnlg1565	4.09	204.19
bnlg1890	4.11	200.63
bnlg1006	5.00	229.38
phi396160	5.02	301.03
phi109188	5.03	161.52
bnlg653	5.04	156.23
phi330507	5.04	131.59
phi331888	5.04	130.72
bnlg1208	5.04	118.88
bnlg1892	5.04	147.78
bnlg2323	5.04	198.10

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Public Name of Marker	bin #	PH6WG base pairs
phi333597	5.05	213.82
phi085	5.06	250.16
bnlg1118	5.07	82.56
bnlg1346	5.07	189.21
bnlg1711	5.07	176.65
phi423796	6.01	135.33
bnlg1422	6.01	215.79
phi389203	6.03	306.15
phi452693	6.04	123.30
phi445613	6.05	96.95
umc1413	6.05	302.96
bnlg1174	6.05	218.72
umc1463	6.06	298.29
phi299852	6.07	117.30
phi364545	6.07	131.46
bnlg1740	6.07	230.27
bnlg1759	6.07	125.81
phi070; umc1063	6.07	83.47
bnlg2132	7.00	200.40
umc1159	7.01	227.99
bnlg1292	7.01	138.74
bnlg1094	7.02	157.39
phi034	7.02	119.55
bnlg1070	7.03	145.56
bnlg2271	7.03	233.69
phi328175	7.04	98.87
phi260485	7.05	318.75
phi051	7.05	138.57
phi069	7.05	195.31
phi116	7.06	164.92
bnlg1194	8.02	174.99
phi100175	8.03	144.28
bnlg1863	8.03	212.97
bnlg2082	8.03	172.52
phi115	8.03	290.66
phi121	8.03	93.90
bnlg2046	8.04	326.12
bnlg1176	8.05	217.94
bnlg1152	8.06	148.42
bnlg1031	8.06	288.71
bnlg1065	8.07	239.09
bnlg1828	8.07	185.65
bnlg1056	8.08	110.49
bnlg1940	8.08	214.68
phi015	8.08	79.78
phi233376	8.09	152.17
bnlg1810	9.01	197.34

Public Name of Marker	bin #	PH6WG base pairs
bnlg2122	9.01	237.09
umc1037	9.02	195.04
bnlg1012	9.04	157.54
phi032	9.04	236.63
phi108411	9.05	122.38
phi236654	9.05	117.08
bnlg619	9.07	272.68
phi041	10.00	202.94
phi96342	10.02	249.64
phi059	10.02	143.60
bnlg1037	10.03	117.77
bnlg1079	10.03	170.34
bnlg1655	10.03	126.51
phi050	10.03	83.27
phi301654	10.04	128.33
phi062	10.04	157.81
phi323152	10.05	141.15
bnlg1074	10.05	183.85
bnlg1028	10.06	127.73
bnlg1185	10.07	151.61
bnlg1450	10.07	177.71
bnlg1839	10.07	195.90
phi109642	2.03/2.04	137.81
bnlg1720	1.09/1.10	236.44
phi448880	9.06/9.07	176.99

What is an "Essentially D rived Variety"?

The concept of essentially derived variety was introduced into the 1991 Act of the UPOV Convention in order to avoid plagiarism through mutation, multiple back-crossing and to fill the gap between Plant Breeder's Rights and patents, gap which was becoming important due to the development of the use of patented genetic traits in genetic engineering.

An essentially derived variety is a variety which is distinct and predominantly derived from a protected initial variety, while retaining the essential characteristics of that initial variety.

As indicated as an example in the UPOV Convention, essentially derived varieties may be obtained by the selection of a natural or induced mutant, or of a somaclonal variant, the selection of a variant individual from plants of the initial variety, back-crossing, or transformation by genetic engineering.

The commercialization of an essentially derived variety needs the authorization of the owner of the rights vested in the initial variety.

The concept of essentially derived variety does not at all abolish the Breeder's Exemption, as free access to protected plant varieties for breeding purposes is maintained. It is not a threat to biodiversity. On the contrary, it favors biodiversity, encouraging breeders developing and marketing original varieties.

Appendix E

UPOV Publication No. 644(E), Section 1

INTERNATIONAL CONVENTION

FOR THE

PROTECTION OF NEW VARIETIES OF PLANTS

of December 2, 1961, as revised
at Geneva on November 10, 1972,
on October 23, 1978, and
on March 19, 1991

adopted by the Diplomatic Conference
on March 19, 1991

reproduced from UPOV Publication No. 438(E)
issue No. 63 of "Plant Variety Protection"

1991 Act of the Convention

Article 12
Examination of the Application

Any decision to grant a breeder's right shall require an examination for compliance with the conditions under Articles 5 to 9. In the course of the examination, the authority may grow the variety or carry out other necessary tests, cause the growing of the variety or the carrying out of other necessary tests, or take into account the results of growing tests or other trials which have already been carried out. For the purposes of examination, the authority may require the breeder to furnish all the necessary information, documents or material.

Article 13
Provisional Protection

Each Contracting Party shall provide measures designed to safeguard the interests of the breeder during the period between the filing or the publication of the application for the grant of a breeder's right and the grant of that right. Such measures shall have the effect that the holder of a breeder's right shall at least be entitled to equitable remuneration from any person who, during the said period, has carried out acts which, once the right is granted, require the breeder's authorization as provided in Article 14. A Contracting Party may provide that the said measures shall only take effect in relation to persons whom the breeder has notified of the filing of the application.

CHAPTER V
THE RIGHTS OF THE BREEDER

Article 14
Scope of the Breeder's Right

(1) [Acts in respect of the propagating material] (a) Subject to Articles 15 and 16, the following acts in respect of the propagating material of the protected variety shall require the authorization of the breeder:

(i) production or reproduction (multiplication),

(ii) conditioning for the purpose of propagation,

(iii) offering for sale,

(iv) selling or other marketing,

(v) exporting,

(vi) importing,

(vii) stocking for any of the purposes mentioned in (i) to (vi), above.

(b) The breeder may make his authorization subject to conditions and limitations.

(2) [Acts in respect of the harvested material] Subject to Articles 15 and 16, the acts referred to in items (i) to (vii) of paragraph (1)(a) in respect of harvested material, including entire plants and parts of plants, obtained through the unauthorized use of propagating material of the protected variety shall require the authorization of the breeder, unless the breeder has had reasonable opportunity to exercise his right in relation to the said propagating material.

(3) [Acts in respect of certain products] Each Contracting Party may provide that, subject to Articles 15 and 16, the acts referred to in items (i) to (vii) of paragraph (1)(a) in respect of products made directly from harvested material of the protected variety falling within the provisions of paragraph (2) through the unauthorized use of the said harvested material shall require the authorization of the breeder, unless the breeder has had reasonable opportunity to exercise his right in relation to the said harvested material.

(4) [Possible additional acts] Each Contracting Party may provide that, subject to Articles 15 and 16, acts other than those referred to in items (i) to (vii) of paragraph (1)(a) shall also require the authorization of the breeder.

(5) [Essentially derived and certain other varieties] (a) The provisions of paragraphs (1) to (4) shall also apply in relation to

(i) varieties which are essentially derived from the protected variety, where the protected variety is not itself an essentially derived variety,

(ii) varieties which are not clearly distinguishable in accordance with Article 7 from the protected variety and

(iii) varieties whose production requires the repeated use of the protected variety.

(b) For the purposes of subparagraph (a)(i), a variety shall be deemed to be essentially derived from another variety ("the initial variety") when

(i) it is predominantly derived from the initial variety, or from a variety that is itself predominantly derived from the initial variety, while retaining the expression of the essential characteristics that result from the genotype or combination of genotypes of the initial variety,

(ii) it is clearly distinguishable from the initial variety and

(iii) except for the differences which result from the act of derivation, it conforms to the initial variety in the expression of the essential characteristics that result from the genotype or combination of genotypes of the initial variety.

(c) Essentially derived varieties may be obtained for example by the selection of a natural or induced mutant, or of a somaclonal variant, the selection of a variant individual from plants of the initial variety, backcrossing, or transformation by genetic engineering.

Article 15
Exceptions to the Breeder's Right

(1) [Compulsory exceptions] The breeder's right shall not extend to

(i) acts done privately and for non-commercial purposes,

(ii) acts done for experimental purposes and

(iii) acts done for the purpose of breeding other varieties, and, except where the provisions of Article 14(5) apply, acts referred to in Article 14(1) to (4) in respect of such other varieties.

(2) [Optional exception] Notwithstanding Article 14, each Contracting Party may, within reasonable limits and subject to the safeguarding of the legitimate interests of the breeder, restrict the breeder's right in relation to any variety in order to permit farmers to use for propagating purposes, on their own holdings, the product of the harvest which they have obtained by planting,

Attorney Docket No. 1329

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Brian Douglas Swanson	Date:	March 14, 2003
Serial No.:	09/759,704	Group Art Unit:	1638
Filed:	January 12, 2001	Examiner:	David H. Kruse
For:	"INBRED MAIZE LINE PH6WG"		

Assistant Commissioner for Patents
Washington, D.C. 20231

RULE 132 DECLARATION
OF
DR. STEPHEN SMITH

Sir:

I, Stephen Smith, PhD., do hereby declare and say as follows:

1. I am skilled in the art of the field of the invention. I have a Ph.D. in Biochemical Systematics and Taxonomy of Maize and its Wild Relatives from Birmingham University. I have a M.Sc. in the Conservation and Utilization of Plant Genetic Resources from Birmingham University. I have a Bachelor of Science degree in Plant Sciences from London University. Since 1977 I have been engaged in the development, study and application of molecular markers to genetics, measuring genetic diversity and tracking pedigrees. I commenced this work at North Carolina State University as a post-doctoral research fellow. I have continued my engagement in these studies during my employment by Pioneer Hi-Bred from 1980 until the present. These studies have resulted in numerous scientific articles that have appeared in peer reviewed scientific literature.

2. This declaration is in response to the Examiner's rejection under, 35 U.S.C. § 102(b) as anticipated by or, in the alternative, under 35 U.S.C. § 103(a) as obvious over Hoffbeck (U.S. Patent No. 5,463,173).

3. I have conducted an analysis of SSR marker data for inbred PH6WG and the inbred cited as prior art, PHR61. Out of a total of 324 SSR loci examined, which allowed a sampling of each chromosome, there are 122 markers that show differences between PH6WG and PHR61. This represents a difference for 38% for the markers tested. Of

Appendix F

these 122 markers, 34 were greater than 50 cM in distance, or unlinked on the genetic map.

4. Upon crossing PH6WG to any other maize line and selfing successive filial generations, one would within the realm of what is statistically possible, obtain a progeny inbred maize line that retains genetic contribution from PH6WG. Assuming that (i) the cited prior art is used as the maize line to which PH6WG is crossed, (ii) that the only difference between PH6WG and PHR61 are these 122 markers, and (iii) that all markers within a 50 cM distance will segregate together, then the odds of obtaining a PH6WG progeny inbred that is the same as PHR61 after one cycle of breeding, is 1 in 2^{34} or 1 in 17,179,869,180. Statistically it is extremely unlikely that a PH6WG progeny, after one cycle of breeding, would be the same as PHR61.

5. Further, the assumptions made above vastly overstate the likelihood of breeding PHR61 from PH6WG. For example, it is common practice in quantitative genetics to determine the relation of plants by differences in markers. In doing so, one extrapolates that a percentage difference in markers is indicative of a difference in the whole genome. To assume that the only differences between PH6WG and PHR61 are for these 122 markers, when 122 markers constitute 38% of the 324 SSR loci examined, is a gross and unrealistic assumption. Further the current maize genetic map only has approximately sixty 50cM units, so by applying this limitation the maximum number of independently segregating loci one could obtain, using the most different maize lines that could ever be found, is sixty. These assumptions result in an over estimate of the odds of breeding PHR61 from PH6WG.

6. Given the difference in molecular markers between PH6WG and PHR61, it is my expert opinion that PH6WG and PHR61 are very distinct inventions. It is also my expert opinion that, within the realm of what is statistically possible, any progeny of PH6WG developed through crossing PH6WG with another plant will be distinct from PHR61. Given the facts and based on my education and scientific experience, I believe that the invention as claimed is not obvious nor anticipated by Hoffbeck (U.S. Patent No. 5,463,173).

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

09/759,704

Date: Mar. 14th 2003 By: Stephen Smith

Stephen Smith